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The Immunological Properties of Proteins Treated with Di-2-chloroethylmethylamine

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When proteins are treated with 'sulphur mustard' (mustard gas; di-2-chloroethyl sulphide) they undergo a slight modification of their immunological properties, and the injection of mustard gas-treated proteins into rabbits may lead to the formation of antibodies capable of reacting specifically with any mustard gas-treated protein (Berenblum & Wormall, 1939; Boursnell, Francis & Wormall, 1946*a*). It was suggested (Berenblum & Wormall, 1939) that acquired hypersensitivity to mustard gas might be partly due to an immunological change in the tissue proteins exposed to mustard gas, i.e. to the formation of 'foreign' proteins and subsequent production of specific antibodies. This view was later supported by the induction of hypersensitivity in laboratory animals by the application of mustard gas (Holiday, 1942; Kidd & Landsteiner, 1944; Moore, 1944). More recently, Fleming, Moore & Butler (1949) have shown that in the presence of phosphate buffer at pH 7·5 or 9, mustard gas reacts with proteins to give complexes which contain residues of diethyl sulphide, phosphate and protein, and that when the mustard gas-protein complexes prepared with added phosphate at pH 8–9 are injected into rabbits they give rise to strongly precipitating antisera.

The nitrogen mustards also react with a few enzymes and other proteins. Di-2-chloroethylmethylamine ($CH_3.N.(CH_2.CH_2Cl)_2$), e.g., strongly inhibits hexokinase (Dixon & Needham, 1946), cholinesterase (Thompson, 1947; Adams & Thompson, 1948), and choline oxidase and choline acetylase (Barron, Bartlett & Miller, 1948). It has been shown that this nitrogen mustard reacts with the amino, carboxyl, sulphydryl, sulphide and pyridine groups of proteins (cf. Fruton, Stein & Bergmann, 1946; for other references see Gilman & Philips, 1946; Boyland, 1948; Philips, 1950; Karnofsky, 1950) and that it rapidly inactivates the protein components of haemolytic complement (Watkins & Wormall, 1948, 1952).

In view of the fairly widespread clinical use of the nitrogen mustards for the treatment of Hodgkin's and certain other neoplastic diseases, we decided to study the immunological properties of proteins treated with the nitrogen mustards. One of the objects of this work was to determine whether antibodies specific for nitrogen mustard-treated proteins could be produced by the repeated injection of nitrogen mustard or nitrogen mustard proteins; any antibody response of this type might indicate the possibility of hypersensitivity occurring in patients subjected to prolonged treatment with the nitrogen mustards. It was also hoped that these immunological and chemical studies might yield information about the mode of action of the nitrogen mustards on amino-acids, peptides and proteins.

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EXPERIMENTAL

Nitrogen mustard (*NM*). The only NM used in these experiments was di-2-chloroethylmethylamine hydrochloride (code no. HN2). After the addition of the solid to protein solutions, the mixtures were shaken gently until the solid had dissolved. When NM was intravenously injected into rabbits, the solid was quickly dissolved in a small volume of 0·9% (w/v) NaCl and the solution injected immediately.

Ovalbumin. Hen ovalbumin, prepared by $(NH_4)_2SO_4$ precipitation, was recrystallized three times (cf. Cole, 1933).

Antigens

The general method used for the preparation of the NM-treated proteins (NM proteins) was as follows. The NM was added, in portions, to the protein solution kept at 37°, and 5% (w/v) Na_2CO_3 was added to the mixture, when required, to maintain the pH at about 7·5, or in a few experiments 7·5–8·0. One-quarter of the NM was added at the start and the same amount about 4 hr. later, and this treatment was repeated the next day. On the third day the mixture was again kept for 8 hr. at 37°, this time without further addition of NM. The mixtures were kept overnight in the refrigerator during this period.

With a few exceptions (the antigens designated *A*, *C* and *E* below) the products were then dialysed against frequently changed 0·9% NaCl for 48 hr. at 4°, to remove transformation or 'hydrolysis' products of NM. Dilute HCl or Na_2CO_3 was added where necessary to bring to pH 7·4, Merthiolate (0·01%) was added as a preservative, and the solutions were stored at 0–4°. Early in these investigations we observed that the NM used has a very powerful bactericidal action, but we continued to add Merthiolate to the antigen solutions

prepared for storage since it was considered possible that the bactericidal activity of the nitrogen mustard might be due to some of its unstable cyclic transformation products.

When most of this work had been completed, the paper by Fleming *et al.* (1949) appeared, describing the effect of phosphate on the reaction between ordinary mustard gas and proteins. Since it seemed possible that phosphate might similarly affect the reaction between NM and proteins, a few further experiments were made with antigens prepared in the presence of extra phosphate.

The following antigens were prepared by the general method described above, with occasional slight modification (detailed below). These antigen preparations contained hydrolysis products of NM. No attempt was made in the preparation of antigens *A*, *C* and *E* to remove these impurities since it was thought that the treatment involved, for example, dialysis or the precipitation of the protein complexes by ethanol, might split NM-protein linkages. Control tests showed that any hydrolysis products of nitrogen mustard likely to be present after these antigen preparations had been kept for several weeks would not in any way affect precipitin or complement-fixation tests.

Antigens for immunization

(i) *NM ovalbumin.* Preparation *A* was made by the action of NM (1·5 g.) on a solution of crystalline ovalbumin (100 ml. containing 7·6 g. of protein). Preparation *B* (extra phosphate) was made similarly, but 0·5M-phosphate buffer of pH 8·0 (50 ml.) was added to the ovalbumin solution before addition of the NM.

(ii) *NM horse serum proteins.* Preparation *C*: normal horse serum (100 ml.) was treated with NM (0·80 g.). Preparation *D* was made similarly, with 0·5M-phosphate buffer of pH 8·0 (50 ml.) added to the horse serum.

Test antigens

In the precipitin and complement-fixation tests, the following antigens were used in addition to the above-described immunizing antigens. All the test-antigen solutions had a pH of about 7·4.

(i) *NM rabbit serum proteins.* Preparation *E*: normal rabbit serum (10 ml.) was treated with NM (80 mg.). The same method was used for preparations *F* (extra bicarbonate) and *G* (extra phosphate), except that additional buffers, 0·5M-$NaHCO_3$-H_2CO_3 (pH 7·5) (5 ml.) and 0·5M-phosphate (pH 8·0) respectively, were added to the rabbit serum.

(ii) *NM gelatin.* Preparation *H*: NM (300 mg. added in three 100 mg. portions at intervals of a few hours) was added to a mixture of a gelatin solution (500 mg. in 30 ml.) and 0·5M-$NaHCO_3$ (50 ml.) and the solution kept at 37° and pH 7·5–8·0 for 48 hr. In the preparation of the 'phosphate-antigen' (*J*) 0·5M-phosphate buffer (20 ml.) was used instead of $NaHCO_3$.

NM-treated amino-acids. Many attempts were made to prepare NM-amino-acid derivatives for use in precipitin-inhibition tests. Details of the methods used are given in the text.

Methods

Nitrogen determinations. Total N was determined by the micro-Kjeldahl method, using the Markham (1942) distillation apparatus. The Van Slyke (1912) volumetric method was used for the determination of α-amino N.

Immunization. In view of the weak specific antibody response following the injection of proteins treated with sulphur mustards (Berenblum & Wormall, 1939; Boursnell *et al.* 1946*a*), we decided to use several immunization methods in our attempts to get antibodies specific for NM proteins.

(*a*) *Intravenous injection of NM.* Solutions of NM hydrochloride in 0·9% NaCl were injected into the ear veins of rabbits on 4 successive days. After an interval of 12 days each rabbit received a further course of four similar injections, except no. 572 which received two injections only, because of badly damaged ear veins. The amounts of NM hydrochloride injected at each injection were 0·1 mg. (rabbits nos. 523 and 563), 0·2 mg. (nos. 614 and 619) and 0·3 mg. (nos. 572 and 576) per kg. of body weight. Blood samples were taken 7 and 14 days after the final injections, and the sera were tested for antibodies capable of reacting with NM proteins.

(*b*) *Intramuscular injection of alum-precipitated antigens.* The protein complexes were precipitated by potassium alum at pH about 6·5, as recommended by Proom (1943), and each rabbit received an intramuscular injection of 5 ml. of a suspension (containing about 17 mg. of protein/ml.) into each hind leg. A course of three injections was given, at intervals of 12–14 days, and the sera were tested 10 days after each injection.

(*c*) *Intraperitoneal injections.* The antigen solution (5 ml.) was injected intraperitoneally, and each rabbit, except no. 686 which died 10 days after the third injection, received five injections at intervals of 9–12 days. Precipitin tests were made on the blood serum samples taken 9 or 10 days after the third and subsequent injections.

In all, twenty-seven rabbits were immunized by methods (*b*) and (*c*), and details of the antigens and the mode of immunization are given in Table 1. The antisera were filtered through Berkefeld filters and stored in sterile tubes at 0–4°.

Table 1. *Details of immunizations with NM proteins*

(IM, intramuscular injections of the alum-precipitated antigen; IP, intraperitoneal injections.)

Group	Rabbits (nos.)	Immunizing antigen	Mode of injection
1	731, 744, 746, 747	NM horse serum (preparation *C*)	IM
2	734, 750, 751, 754	NM ovalbumin (preparation *A*)	IM
3	556, 654, 671, 708, 727	NM ovalbumin (preparation *A*)	IM
4	795, 809, 841	NM ovalbumin* (preparation *B*)	IP
5	679, 681, 686	NM horse serum* (preparation *D*)	IP
6	689, 719, 837, 840	NM ovalbumin* (preparation *B*)	IM
7	666, 692, 797, 854	NM horse serum* (preparation *D*)	IM

* Antigens prepared in the presence of extra phosphate.

Precipitin tests. These were made as described previously (Hopkins & Wormall, 1933). The tests with NM gelatin as test antigen were made at room temperature.

Precipitin-inhibition tests. The substance or mixture being tested for inhibitory power was dissolved in 0·9% NaCl to give a 1% (w/v) solution, or, with sparingly soluble substances, a saturated solution (at room temperature) was prepared. The solution was neutralized and varying amounts (1–4 vol.) were added to the antigen solution (1 vol.); 0·9% NaCl was then added (to give a total of 5 vol.). Antiserum (2 vol.) was added and the mixtures were shaken and kept at 37°. The extent of precipitation was noted after 15 min., 1 and 3 hr.

Complement-fixation tests. These were carried out as described previously (Berenblum & Wormall, 1939), but with a sensitized sheep erythrocyte system.

RESULTS

Intravenous injection of NM into rabbits

The sera of six rabbits which were given frequent injections of NM contained no antibodies capable of precipitating NM ovalbumin. Thus no evidence was obtained that the injection of NM leads to the production of antibodies specific for NM proteins, even when the amount injected is appreciably greater than that injected into patients suffering from Hodgkin's and some other neoplastic diseases. The amount of NM injected into man varies very considerably, but a widely adopted method is a course of injections of 0·1 mg./kg. of body weight on each of 4 successive days, with a second similar course some weeks or months later. In our experiments doses three times as large as these, with a second course after an interval of 12 days, failed to elicit the formation of antibodies specific for NM proteins.

Injection of NM proteins

Although antibodies specific for NM proteins could not be detected in the serum of rabbits following the injection of NM, the possibility of antibody production in the tissues of these injected animals cannot be excluded. We decided, therefore, to study the effect of injecting preformed NM proteins. Horse serum proteins and ovalbumin, previously treated with NM, were injected intraperitoneally or intramuscularly (as the alum-precipitated antigen) into several groups of rabbits, and the sera of these animals were tested at intervals for antibodies capable of reacting, in precipitin and complement-fixation tests, with various NM-proteins.

Some of the injected rabbits had no detectable 'NM protein-specific' antibodies in their serum at any time during the long immunization; all produced antibodies which precipitated the injected antigen or the corresponding untreated protein (horse serum proteins or ovalbumin), showing that the injected complexes were fully antigenic. Thus treatment with considerable amounts of NM at pH 7·5–8 does not destroy the antigenicity of ovalbumin or the serum proteins.

The sera of seven of the twenty-seven injected rabbits gave, at some stage in their immunization, weak but significant precipitin reactions with NM proteins different from those used for immunization. For example, the injection of NM horse serum proteins occasionally gave rise to antibodies which

Table 2. *Precipitin reactions with antisera to proteins treated with nitrogen mustard (NM)*

(Precipitin reactions are recorded, as follows: −, (no reaction); tr., (trace); ±, +, +±, ++ etc., in increasing degrees of precipitation.)

Antigen	Concentration of antigen solution (%)	Antisera against: NM ovalbumin No. 751	NM ovalbumin No. 754	NM horse serum proteins No. 746	NM horse serum proteins No. 747
NM ovalbumin	0·25	+++	+++	−	−
	0·05	+++	+++	−	−
	0·01	++	++	±	−
	0·002	−	tr.	−	−
NM horse serum proteins	0·25	−	−	+±	+±
	0·05	±	−	++±	++±
	0·01	±	±	+	+
	0·002	−	−	tr.	−
NM rabbit serum proteins	0·25	−	−	−	−
	0·05	tr.	tr.	±	±
	0·01	−	−	tr.	tr.
	0·002	−	−	−	−
Ovalbumin	0·25	+±	+±	−	−
	0·05	±	±	−	−
	0·01	tr.	tr.	−	−
	0·002	tr.	tr.	−	−
Horse serum proteins	0·25	−	tr.	+±	+±
	0·05	tr.	−	++±	++±
	0·01	−	−	+±	+±
	0·002	−	−	tr.	−

gave precipitates with NM ovalbumin or NM rabbit serum proteins, but not with NM gelatin. Typical results of these reactions are given in Table 2. No evidence was obtained that the specific antibody response depended on the particular NM protein injected or on the mode of immunization. The results with the NM protein antigens prepared in the presence of phosphate were not significantly different from those obtained with antigens prepared without added phosphate.

Complement-fixation tests fully confirmed this observation that antibodies specific for NM proteins were occasionally produced in the injected rabbits. Typical results (Table 3) showed that the sera of some of the rabbits injected with NM ovalbumin reacted with heterologous NM proteins, e.g. NM horse serum proteins. No complement fixation was obtained when normal horse serum proteins were used as the antigen in these tests with antisera to NM ovalbumin.

Table 3. *Complement-fixation tests with antisera to NM ovalbumin*

(Mixtures of 0·5 ml. of the diluted antigen (of concentration recorded in column 1), 0·10 ml. of antiserum (previously heated at 56° for 20 min. to inactivate any complement present) and 0·10 ml. of dilute guinea pig serum (1 vol. of serum plus 4 vol. of 0·9% NaCl) were kept at room temperature for 1 hr. After the addition of 0·5 ml. of a 3% suspension of sensitized sheep erythrocytes to each tube, the mixtures were shaken and kept at 37°, and the extent of haemolysis was noted at intervals. The readings recorded in this table were those after 1 hr. Degrees of haemolysis; 0, none; tr., trace; 1, 2 and 3, increasing degrees of haemolysis; 4, complete haemolysis.)

Concentration of antigen (NM horse serum proteins) (mg./100 ml.)	Degree of haemolysis	
	Antiserum no. 750	Antiserum no. 734
250	4	4
83	4	4
28	3	4
9·3	2	3
3·1	tr.	2
1·0	0	1
0·34	0	0
0·11	0	0
0·038	0	0
0·013	0	tr.
0·004	tr.	1
None (control)	4	4

Attempts to obtain specific inhibition of the reaction between NM proteins and their antibodies

Our immunological and chemical evidence suggests that the slight change in the immunological properties of proteins effected by NM is due to the combination of NM and the protein. It is conceivable, therefore, that a suitable NM amino-acid or NM peptide might be found which would specifically inhibit the NM protein antigen-antibody reaction, for example the precipitin reaction between NM ovalbumin and antibodies to NM horse serum globulins. Specific inhibition of this type would, of course, provide useful information about the nature of the protein groups to which the NM becomes attached.

Attempts were made to isolate products formed by the action of NM on various amino-acids, and in these experiments, as in the preparation of NM protein antigens, we kept the conditions as near as possible to the physiological, namely temperatures not exceeding about 37° and a pH of 7·5–8·5. Fruton, Stein & Bergmann (1946) describe the reduction of free amino nitrogen which occurs when solutions of various amino-acids are shaken with NM at pH 8–8·5 and 25°, and they isolated from NM plus phenylalanine a reaction product to which they gave the formula

$$CH_3N(CH_2.CH_2.NH.CH(CH_2.C_6H_5).COOH)_2.$$

Our efforts to prepare this pure substance have, unfortunately, been unsuccessful, and so have our attempts to obtain pure NM derivatives of glycine, lysine and cysteine. The method we used was essentially that described by Fruton, Stein & Bergmann (1946), and although with all these amino-acids we observed a considerable reduction (24–41%) in the free amino nitrogen and a complete loss of free SH when cysteine was used, we were unable to separate crystalline reaction products; in some instances we attempted, without success, to separate the NM amino-acid derivatives as copper salts, a method found satisfactory for a mustard gas derivative of glycine (Boursnell, Francis & Wormall, 1946*b*). Since, however, the relatively crude products differed from the original amino-acids in total nitrogen and free amino-nitrogen contents, it was decided to find out whether they specifically inhibited the NM protein antigen-antibody reaction.

In a further search for inhibitory substances and as part of a more general study of the action of NM on proteins, we investigated the action of NM on nucleic acid and glutathione.

(*a*) Yeast nucleic acid (British Drug Houses) was purified by dissolving the sodium salt in water and precipitating the acid at pH 1·5–3·0. After it had been washed successively with ethanol and ether and dried at 50°, the product (0·5 g.) was dissolved in 0·5 M-$NaHCO_3$-H_2CO_3 buffer of pH 7·5 (20 ml.) and mixed with a freshly prepared neutralized solution of NM (0·25 g.) in about 4 ml. of water. The mixture was kept at room temperature for 24 hr. and three fractions were separated by treatment with N-HCl: (*A*) a light-brown solid (0·43 g.) precipitated over the range pH 2–4, (*B*) an almost white solid (0·06 g.) precipitated at pH 1–2, and (*C*) a white solid (0·03 g.) precipitated from the mixture at pH 1, after the removal of fraction (*B*), by adding excess of ethanol. All these fractions were washed with ethanol and then with ether, and finally dried *in vacuo*. They

were not sufficiently pure for complete analysis, but N and P determinations were made on the two largest fractions (*A*) and (*B*); these had N/P ratios of 2·06 and 1·95 respectively, compared with a ratio of 1·79 for the untreated purified nucleic acid.

(*b*) A freshly prepared solution of NM (400 mg.) in 0·5 M-$NaHCO_3$-H_2CO_3 buffer of pH 7·5 (5 ml.) was added to a solution of glutathione (300 mg.) in $NaHCO_3$ buffer (5 ml.) and the mixture was kept at room temperature. After 30 min. there was no free SH detectable by nitroprusside, and in the course of 48 hr. there was a 48 % reduction in the free amino nitrogen of the solution. The mixture was then concentrated to a syrup *in vacuo* over H_2SO_4 at room temperature, and the crystalline material which separated was rubbed with acetone to give a flocculent powder which was washed with acetone and dried. The remaining syrup was kept for some time *in vacuo* over conc. H_2SO_4, and the gelatinous solid obtained was rubbed with acetone, giving a powder which was washed with acetone and dried. In view of the inability of these products to inhibit the NM protein serological systems, no attempts have so far been made to purify and identify them.

Precipitin inhibition tests with the above-mentioned NM-treated amino-acids, nucleic acid and glutathione showed that these products had no specific inhibitory effect on the NM protein antigen-antibody system. Where inhibition occurred it was of a non-specific nature, for the product concerned inhibited unrelated precipitin reactions. No inhibition was obtained with some 'hydrolysis' products of NM (namely di-2-hydroxyethylmethylamine, 2-chloroethyl-2-hydroxyethylmethylamine and the dimer, *NN'*-di(2-chloroethyl)-*NN'*-dimethylpiperazinium chloride), or with the following NM derivatives; a crystalline compound formed by the interaction of NM and di-2-hydroxyethylmethylamine (Fruton, Stein, Stahmann & Golumbic, 1946) and the hexamethylenetetraminium derivative formed by the action of NM on hexamethylenetetramine (Gurin, Deluva & Crandall, 1947).

The stability of the NM protein complexes

It was not possible in these investigations to determine the number of NM residues present in the NM protein complexes, and indeed this could only be achieved in later investigations with ^{15}N-labelled NM (Burnop, Richards, Watkins & Wormall 1951). A few preliminary qualitative experiments were made, however, to determine the stability of the linkages between the NM and proteins, and whether the treatment of serum proteins with NM produces protein derivatives which are precipitated, as are the corresponding mustard gas sulphone proteins, by the addition of dilute acid.

Dialysis of NM horse serum proteins for 4 days at 4° against frequently changed 0·9 % NaCl, or their maintenance in acid or alkaline solution (over the range pH 3–9) for 16 hr. at 37° and subsequent dialysis against 0·9 % NaCl at 4°, effected no significant loss of the power of these NM proteins to give precipitates with antisera to NM ovalbumin. A slight loss of precipitability occurred in solutions at pH 11 kept for 16 hr., but this reduction was most probably due to denaturation rather than removal of NM groups, since there was a corresponding decrease in the capacity of the NM protein complex to precipitate antibodies to untreated horse serum globulins.

With regard to precipitation by acid, it was found that the NM serum proteins, prepared by the action at pH 7·5 of either freshly prepared aqueous solutions of NM or NM solutions which had been allowed to hydrolyse (for example, at 37° for 4 hr. followed by 18 hr. at 4°), could not be precipitated by adding dilute acid. In this respect, therefore, the action of NM resembles that of 'sulphur mustard' and differs from that of mustard gas sulphone.

DISCUSSION

The extensive work of Landsteiner and his colleagues (for the literature, see Landsteiner, 1936, 1945) has shown that many simple organic compounds produce, when introduced into the animal body, a condition of hypersensitivity because they react with the body proteins to form immunologically foreign conjugates. Induced hypersensitivity to ordinary mustard gas has been explained in this way (Berenblum & Wormall, 1939), and if NM combines firmly with proteins the risk of producing allergic reactions by injecting nitrogen mustard must be considered.

Our investigations show that when the serum proteins and ovalbumin are treated with nitrogen mustard under physiological conditions of pH and temperature, these proteins undergo modifications in their immunological properties. The complexes formed produce antibodies specific for NM-treated proteins in some, but not all, of the injected rabbits. This alteration in the immunological properties of the proteins is, however, very slight, since specific antibody formation was only occasionally produced. NM undoubtedly combines firmly with proteins, as was indicated by the investigations described here and more fully established by our quantitative studies with nitrogen mustard labelled with ^{15}N (Burnop *et al.* 1951), and it is surprising that the specific antibody production following the injection of NM-treated protein is not stronger and more frequent. The specific antisera produced by the injection of sulphur mustard-proteins were not very potent (Berenblum & Wormall, 1939; Boursnell *et al.* 1946*a*), but they were more powerful than the antisera to NM proteins produced in the experiments described here, and they were obtained in a higher percentage of injected animals.

This infrequency of specific antibody production following the injection of NM proteins may be due to the fact that relatively few NM residues become attached to each protein molecule, and since some of these groups can be detached by long dialysis (Burnop *et al.* 1951), the injected NM proteins may soon lose many of their determinant NM groups. Another possible explanation is that NM might act on proteins mainly by a cross-linking action. Haddow, Kon & Ross (1948) showed that the cytotoxic activity of a series of halogenoalkylarylamines could be related to the presence of two halogenoalkyl groups in the molecule, and Goldacre, Loveless & Ross (1949) suggested that cross-linking might account for the action of the nitrogen mustards in producing chromosome abnormalities (see also Loveless & Revell, 1949; Loveless, 1951). According to this view two reactive groups are required in the NM molecule to permit it to react at two distant points either on the same fibre or on two contiguous fibres. Cross-linking of this type might explain why NM-treated proteins differ so little immunologically from the unchanged protein, for with a cross-linking mechanism there would be no characteristic determinant grouping projecting from the NM protein molecule.

Whatever the explanation, it appears that the injection of NM or of NM-treated proteins into rabbits rarely leads to the production of antibodies specific for NM proteins and this is supported by clinical evidence. There are apparently few, if any, cases of allergic reactions in man attributable to the intravenous injection of the nitrogen mustards. Furthermore, Hartman, Mangun, Feely & Jackson (1949) found no evidence of antigenic effects when man and dogs were given repeated injections of plasma to which NM had been added as a preservative. On the other hand, Moore & Rockman (1950) have recently found that skin hypersensitivity to one of the nitrogen mustards (di-2-chloroethylethylamine) can be induced in man by a single NM burn or preferably two successive burns with a week's interval between the applications of NM; no evidence was obtained of simple cross-reactions between sulphur mustards and NM in these sensitivity tests. These observations of Moore & Rockman suggest that the possibility of occasional production of hypersensitivity to NM should not be overlooked.

Nitrogen mustards such as di-2-chloroethylmethylamine react with many groups in the protein molecule (cf. reviews by Philips, 1950, and Karnofsky, 1950) and they degrade deoxyribonucleic acid solutions as shown by a decrease in viscosity (Gjessing & Chanutin, 1946; Butler, Gilbert & Smith, 1950; Butler & Smith, 1950). It is not known, however, which parts of the protein molecule are primarily concerned when NM effects its characteristic biological actions, including the slight serological change described in this paper. Our attempts to obtain information on this point by serological inhibition tests have so far been unsuccessful, for we have not been able to obtain any NM amino-acid derivative or any similar preparation which will specifically inhibit the reaction between NM proteins and their antibodies. With the aid of NM labelled with ^{15}N and ^{14}C we are making a further study of the action of NM on proteins and tissue constituents, and it has now been possible to determine the amount of NM which combines firmly under physiological conditions with the serum and other proteins (Burnop *et al.* 1951). Quantitative data about the combination of NM and nucleic acids has also been obtained (Burnop, Richards & Wormall, 1952), and it is hoped that these quantitative studies will throw some light on the immunological results reported here.

SUMMARY

1. Antibodies capable of reacting with NM (nitrogen mustard)-treated proteins could not be detected in the sera of rabbits which had received a series of intravenous injections of di-2-chloroethylmethylamine, one of the nitrogen mustards frequently used therapeutically.

2. The sera of rabbits which had been given a course of injections of NM-treated proteins (horse serum proteins or ovalbumin treated with NM at 37° and pH 7·5–8·0) occasionally contained antibodies capable of reacting specifically with NM proteins, including NM-treated rabbit serum proteins.

3. This specific antibody production was, however, very weak and infrequent. It is concluded that the risk of hypersensitivity following frequent exposure to NM or a series of intravenous injections of the drug, is slight, at least with the rabbit.

4. The complexes formed when NM acts on serum proteins or ovalbumin are comparatively stable, and the NM protein does not lose its serological specificity as a result of long dialysis. Attempts to obtain a NM amino-acid compound capable of specifically inhibiting the NM protein antigen-antibody reaction have been unsuccessful.

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The Determination of Plasma Fibrinogen by the Clot-Weight Method

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The clot-weight technique is a convenient method for the determination of plasma fibrinogen. The result is obtained as fibrin with sufficient accuracy for clinical purposes. Nevertheless, certain possible causes of error appear not to have received attention and these have been investigated.

METHODS

Plasma. 9 vol. of human blood are received from a paraffined syringe into 1 vol. of sodium citrate solution containing 3·8 g./100 ml. and the mixture is centrifuged at 2000 rev./min. for 30 min.

Technique of fibrinogen determination. The citrated plasma is diluted with an approximately equal vol. of 0·025 M-$CaCl_2$ solution and allowed to stand at 37°. The fibrin is wound off on to a wooden applicator until no further clot forms, washed in distilled water, rolled off the rod, drained between filter papers, pulled out thin (when of sufficient bulk), dehydrated first in acetone and then in air at 105° for 3 hr., and weighed.

From the haematocrit of the citrated blood the calculation is made as follows:

$$\text{Plasma fibrinogen content} = \frac{\text{clot weight}}{\text{sample volume}} \times \frac{\text{volume of citrated plasma in 10 ml. citrated blood sample (from haematocrit)}}{\text{volume of citrated plasma} - \text{volume of citrate}}.$$

This correction ignores the variable effect of the citrate upon the cell-plasma fluid partition, but error from this cause is thought to be small compared with differences between persons.

EXPERIMENTAL

Formal experiments were made to determine the effect of certain variables in technique which in practice it may not always be possible to standardize.

Effect of sample storage. It may not always be possible to make determinations on fresh samples. Beginning, therefore, 3 hr. after the blood was drawn, triplicate determinations were made on 8 successive days (except the fifth) on 5·0 ml. samples from a quantity of plasma stored in glass at about 4°.

The data are shown in Table 1. Analysis of the results showed a significant linear regression increasing on days over the 8 days, but as no effect was apparent until the fourth day after venepuncture, storage errors would appear to be seldom of practical consequence.

Table 1. *Effect of storage on clot weight*

(Weights of clots from 5·0 ml. portions of a sample of plasma stored at 4° and tested 3 hr. after withdrawal (day 0) and on subsequent days for 1 week.)

	Replicate clot weights (mg.)			
Day	1	2	3	Mean
0	14·0	14·0	14·5	14·17
1	14·1	14·0	13·8	13·97
2	14·5	—	14·3	14·37
3	14·3	13·9	14·4	14·20
4	14·7	14·5	14·4	14·53
6	14·7	14·5	14·4	14·53
7	14·4	14·2	14·6	14·40

Effect of sample volume. From one sample of plasma, three replicate determinations were made on subsamples of 5·0, 4·0, 3·0, 2·0 and 1·0 ml. In eight clinical determinations replicates had also been made on unequal volumes.

The data from the formal experiment are shown in Table 2. Comparative analyses were made on the two groups of results. The analysis of the fifteen experimental determinations showed a significant

Table 2. *Effect of sample volume on clot weight*

	Replicate fibrin determinations (mg. fibrin/ml. plasma)			
Sample vol. (ml.)	1	2	3	Mean
5·0	2·08	2·18	2·16	2·14
4·0	2·18	2·15	2·25	2·19
3·0	2·20	2·20	2·20	2·20
2·0	2·25	2·25	2·25	2·25
1·0	2·20	2·20	2·30	2·23

negative linear regression on volume, equivalent to an addition of about 1·3 % to the clot weight for each ml. decrease in volume below 5·0 ml. A similar (but non-significant) association was observed in the eight field determinations, differing from subject to subject, but equivalent, on the average, to an addition of 3·6 % wt./ml. decrease in volume.

Comparison of recalcification with added thrombin for fibrin isolation. As it is theoretically possible that sufficient thrombin to clot its fibrinogen content might not be available from a given sample of plasma, it might be preferable to cause clotting by the addition of thrombin (Jones & Smith, 1930). Nineteen parallel determinations were therefore made on fifteen plasma samples (none from patients known to be suffering from one of the haemorrhagic diseases) by the recalcification technique given above and by diluting with 2 or 3 vol. of saline and then adding thrombin solution until no further clot formed.

Table 3. *Comparison of fibrinogen concentration determined with thrombin and by recalcification*

	Fibrinogen concentration (g./100 ml.)	
Plasma samples	Fibrin obtained by adding thrombin	Fibrin obtained by recalcification
1	0·37	0·37
2	0·74	0·67
3	0·44, 0·45, 0·42	0·46, 0·46, 0·41
4	0·63	0·60
5	0·38	0·36
6	0·37	0·41
7	0·57	0·49
8	0·61, 0·61	0·64, 0·59
9	0·36	0·32
10	0·48, 0·45	0·59, 0·48
11	0·48	0·39
12	0·43	0·46
13	0·29	0·39
14	0·48	0·55
15	0·59	0·59

The data are shown in Table 3. The range of values obtained on the fifteen samples was 0·29–0·74 g./100 ml. and inspection shows no systematic difference between the series at high or at low levels. The mean difference between the two series was 0·006 g./100 ml. in favour of thrombin; $t = 0·042$ ($P > 0·9$). The two methods are clearly equivalent on the basis of this experience.

Effect of dilution before recalcifying. It is usually recommended that the plasma be diluted with 10 or 20 vol. of saline before recalcifying, but this might lead to significant loss from the solubility of fibrin in physiological salt solutions. Six 5·0 ml. replicates were determined by the described procedure in parallel with six similar replicates from the same sample diluted to 45 ml. with 0·9 % (w/v) sodium chloride and then recalcified with 5·0 ml. 0·025 M-calcium chloride.

The data are shown in Table 4. The mean clot weight of the six diluted replicates was 13·73 mg. and of the six undiluted replicates, 14·03 mg.; the difference in means was $0·30 \pm 0·11$, which is significant ($0·02 < P < 0·05$). The lower, diluted value, a decrease of about 2 % on the undiluted value,

suggests that a little fibrin was lost by solution; the described procedure was therefore preferred: in this also, the manipulation of fibrin was found to be easier in the smaller volume.

Table 4. *Effect of dilution on clot weight*

(Weights of clots from twelve 5·0 ml. portions of one sample of plasma: in six instances the recalcified mixture was diluted five times.)

	Clot weight (mg.)	
Replicates	Diluted	Undiluted
1	14·1	14·1
2	13·9	14·0
3	13·6	13·8
4	13·6	14·1
5	13·8	14·1
6	13·4	14·1
Mean	**13·73**	**14·03**

Ash weight of clot. To exclude systematic error from mineral content (Foster & Whipple, 1922), two groups of three clots from 5·0 ml. normal plasma samples were pooled and weighed. Thereafter they were ashed and re-weighed. In neither instance did the combined weight of ash from the pooled clots exceed the limit of balance error ($\pm$ 0·1 mg.).

DISCUSSION

Effect of sample volume. The inverse relation of fibrinogen concentration to the plasma sample volume is curious. It is interesting that a similar inverse relation is apparent in the fibrin dry-weight data of Foster & Whipple (1922) and of Howe (1923), though in neither report does it receive comment; in Howe's data the magnitude of the effect is very small. The error, about 5 % or less per ml. below 5 ml., is obviously small compared with differences expected between persons, if approximately the same volume of plasma, 3–4 ml., is always used.

Effect of sample dilution. The findings suggest that plasma samples should not be much diluted at recalcification, although again the error, a loss of 2 % on clot weight with fivefold dilution of the reaction mixture, is small compared with differences to be expected between persons.

SUMMARY

1. Daily determinations on a stored plasma sample showed a significant increase of clot weight from the fourth day after withdrawal.

2. There is an inverse relation between sample size and determined fibrinogen concentration.

3. Clot weights obtained by recalcification agreed well with those obtained by adding thrombin.

4. Dilution of the sample at recalcification reduced the weight of clot obtained.

5. The ash weight of the clot was negligible.

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Bacterial Arylsulphatase

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It is known that in plants and animals there occur enzymes which catalyse the hydrolysis of sulphuric acid esters. These enzymes, the sulphatases, have recently been reviewed by Fromageot (1950). The sulphatases differ with respect to the type of sulphuric acid ester upon which they act, and they include arylsulphatase (phenolsulphatase), which hydrolyses arylsulphuric acids. The existence of arylsulphatase was first demonstrated by Derrien (1911) who observed that extracts of the shell fish, *Murex trunculus*, hydrolyse potassium indoxyl sulphate. An enzyme with similar activity was later found to be present in *Aspergillus oryzae* by Neuberg & Kurono (1923), and when evidence became available concerning its specificity it was named 'phenolsulphatase' (Neuberg & Simon, 1932).

Recently, the more satisfactory name 'arylsulphatase' has been applied to this enzyme (Robinson, Spencer & Williams, 1951). Arylsulphatase has been shown to be present in the tissues of higher animals (Neuberg & Simon, 1925; Rosenfeld, 1925; Hommerberg, 1931; Huggins & Smith, 1947) and also in neoplasms of the rat (Huggins & Smith, 1947).

Hitherto, no extensive study of the distribution of arylsulphatase among bacteria appears to have been made. An indication that this enzyme is present in some species of bacteria was obtained, however, by Barber, Brooksbank & Kuper (1951). These workers, while using sodium phenolphthalein monoglucuronidate and sodium phenolphthalein diphosphate as substrates for the study of glucuronidase and phosphatase in staphylococci, kindly undertook to test the same organisms for arylsulphatase using, as substrate, potassium phenolphthalein disulphate synthesized by two of us (A.R.M. and L.Y.). Of the 160 strains of *Micrococcus pyogenes* and the seventy-five strains of coagulase-negative staphylococci tested by Barber *et al.* (1951), two showed faint phenolsulphatase activity. More marked arylsulphatase activity was shown, however, by an aerobic sporing bacillus encountered as a contaminant, and by a strain of *Salmonella schottmuelleri*.

In the present investigation, a wide range of bacterial species has been examined for the presence of arylsulphatase and some species have been found to contain the enzyme. The substrate used for testing for the presence of the enzyme was potassium phenolphthalein disulphate. With some bacterial species tests were also carried out using potassium 1-naphthylsulphate as substrate. The synthesis of potassium phenolphthalein disulphate does not appear to have been reported previously and an account of the method used to prepare the compound is given in the present paper, together with a description of some of its properties. A preliminary study of the separation of bacterial arylsulphatase has been made and an account is given of the preparation, from a strain of *Mycobacterium piscium*, of cell-free aqueous extracts with arylsulphatase activity.

A preliminary account of the work described in this paper has been published (Young, Morrison & Whitehead, 1952).

SUBSTRATES USED FOR THE STUDY OF BACTERIAL ARYLSULPHATASE

Potassium phenolphthalein disulphate

Synthesis of potassium phenolphthalein disulphate. Most of the methods used for the synthesis of arylsulphuric acids are based on a procedure developed by Verley (1901) in which chlorosulphonic acid is added slowly to a chilled solution of an organic base in an anhydrous organic solvent. The addition product of the chlorosulphonic acid and the organic base is then allowed to react with the phenol, and the organic base salt of the arylsulphuric acid thus formed is converted to the potassium salt by treatment with aqueous potassium hydroxide. A number of organic bases and solvents have been used in this procedure, e.g. pyridine and carbon disulphide (Verley, 1901), pyridine and chloroform (Czapek, 1914), dimethyl- or diethyl-aniline and carbon disulphide (Burkhardt & Lapworth, 1926). In the present investigation it was found that potassium phenolphthalein disulphate can be prepared in fair yield by the above procedure using pyridine as the base and chloroform as a solvent.

A mixture of 12·5 ml. of dry pyridine and 12·5 ml. of redistilled $CHCl_3$ in a 100 ml. beaker (tall type) was cooled in an ice bath, and while the mixture was stirred, 5·0 ml. of chlorosulphonic acid were run in slowly down the side of the beaker from a tap funnel. To this solution were added quickly, with stirring, 8·0 g. of phenolphthalein in 15 ml. of $CHCl_3$. The ice bath was then removed and the contents of the beaker were stirred for 2 hr., after which they were allowed to stand at room temperature overnight. The beaker was then placed in an ice bath and while its contents were stirred, 80% aqueous KOH solution (w/v) was added until the mixture was just alkaline to litmus (14 ml. of the KOH solution were required). The mixture was stirred for 10 min. after the addition of the KOH solution had been completed and it was then poured, with stirring, into 150 ml. of absolute ethanol. The precipitate which formed was allowed to settle in the refrigerator and was then separated by centrifuging. The solid material was then extracted on a water bath with three 100 ml. portions of a mixture of 80% ethanol and 20% water (v/v). During this process the mixture was kept just alkaline to phenolphthalein by the addition of a few drops of KOH solution. The extracts were cooled separately to room temperature and were then cooled in an ice bath. A heavy precipitate formed in the first extract and was separated by filtration. The filtrate was combined with the second and third extracts, 900 ml. of absolute ethanol were added, and the precipitate which formed was filtered off. The combined precipitates were dissolved in a minimum of water and the compound was salted out by the addition, with vigorous stirring, of 80% aqueous KOH solution (w/v). The *potassium phenolphthalein disulphate* was removed by filtration on a sintered-glass filter, washed with absolute ethanol, and dried. The product weighed 10·5 g. and this corresponded to a yield of 64% based on the amount of phenolphthalein used. The compound was crystallized twice from water and was then dried over P_2O_5 *in vacuo* at room temperature. The product gave negative tests for phenolphthalein, inorganic sulphate, chloride, carbonate and bicarbonate. It gave strong positive tests for phenolphthalein and inorganic sulphate after it had been boiled in N-HCl solution for 1 min. (Found: C, 36·7; H, 2·9; S, 9·8; K, 17·0. $C_{20}H_{13}O_{11}S_2K_3.2H_2O$ requires C, 37·1; H, 2·6; S, 9·9, K, 18·1%. Micro-analyses by Drs Weiler and Strauss, Oxford.) The compound was very soluble in water and was almost insoluble in ethanol. It was obtained as fine colourless needles when precipitated from aqueous solution by the

addition of ethanol, and as rectangular plates when crystallized from water.

Gravimetric determination of phenolphthalein and sulphate liberated by acid hydrolysis of the compound. Phenolphthalein is almost insoluble in dilute HCl solution, and it was thus possible to carry out a gravimetric determination of the amount of phenolphthalein liberated on complete hydrolysis of the potassium phenolphthalein disulphate obtained by the procedure just described. The amount of sulphate liberated on hydrolysis was determined gravimetrically as $BaSO_4$ at the same time. To a solution of 0·06429 g. of the compound dissolved in 1 ml. of water in a small Pyrex filter beaker (fitted with a sintered-glass filter plate) was added 1 ml. of 2N-HCl. The vessel and its contents were heated in a boiling-water bath for 30 min. and then cooled in an ice bath. The crystalline precipitate was filtered off on the sintered-glass plate and washed with three 2 ml. portions of water. The filtrate and washings were collected. The precipitate, after it had been dried to constant weight at 105°, weighed 0·03182 g. It melted at 256–257°, and when it was mixed with phenolphthalein the melting point was unchanged. The sulphate present in the filtrate and washings yielded 0·04674 g. $BaSO_4$. Colorimetric analysis of the filtrate and washings obtained in a similar experiment showed that the phenolphthalein content was negligible (<0·00005 g.). The phenolphthalein and sulphate (as SO_4) liberated corresponded to 49·5 and 29·9%, respectively, of the compound hydrolysed; potassium phenolphthalein disulphate dihydrate requires 49·2% phenolpthalein and 29·7% sulphate.

Colorimetric determination of phenolphthalein liberated by hydrolysis of the compound. In all experiments other than that just described, a colorimetric method was used to determine phenolphthalein liberated by the acid or enzymic hydrolysis of potassium phenolphththalein disulphate. The procedure was as follows. To 5 ml. of the acid (or buffer) solution containing phenolphthalein, NaOH was added from a microburette until the solution was just pink (the concentration of the NaOH solution used was such that the volume required did not exceed 1 ml.). After water had been added to bring the volume of the solution to 6 ml., 4 ml. of buffer solution, pH 10·4 (glycine-NaOH-NaCl, prepared as described by Talalay, Fishman & Huggins (1946)) was added. The red colour of the solution (after dilution, if necessary) was then measured in a Spekker photoeletric absorptiometer (Adam Hilger Ltd.) using an Ilford no. 605 filter. The amount of phenolphthalein present was then calculated by reference to a standard curve obtained using known amounts of phenolphthalein. By this means it was possible to determine as little as 10μg. of phenolphthalein in the original solution.

Experiments were carried out in which 1 ml. portions of a 0·01M-potassium phenolphthalein disulphate solution were heated in a boiling-water bath with 4 ml. portions of either 0·125N-HCl or 1·25N-HCl. Under these conditions it was found that the compound was completely hydrolysed in 15 min. in 0·1N-HCl and in 5 min. in N-HCl.

Similar experiments were carried out at 37°, and under these conditions it was found that in 24 hr. 7·8% of the phenolphthalein was liberated in 0·1N-HCl and 86·4% in N-HCl.

Enzymic hydrolysis of potassium phenolphthalein disulphate. Takadiastase, a commercial enzyme preparation from *Aspergillus oryzae*, shows arylsulphatase activity, and according to Huggins & Smith (1947) the enzyme has an optimum pH of 6·12 at 37° with a substrate of potassium 4-nitrophenylsulphate. In order to study the behaviour of potassium phenolphthalein disulphate on enzymic hydrolysis, experiments were carried out in which takadiastase was allowed to act on the compound at 37°. The enzyme solution was prepared by extracting 2·5 g. of takadiastase diluted with talc (Parke Davis Co. Ltd.) with 50 ml. of water at room temperature. This gave a solution of takadiastase containing 0·37% solid matter, and 1 ml. portions of this were heated in a water bath at 37° with 1 ml. of 0·01M-potassium phenolphthalein disulphate solution and 3 ml. of citrate-phosphate buffer, pH 6·2 (McIlvaine series; Britton, 1942). The amounts of phenolphthalein liberated were determined colorimetrically and the results obtained are shown in Table 1. No liberation of phenolphthalein took place in control experiments in which enzyme solution which had been heated in a boiling-water bath for 15 min. was allowed to act on the substrate for 24 hr. under the conditions just described.

Table 1. *Hydrolysis of potassium phenolphthalein disulphate by takadiastase*

(1 ml. 0·01M substrate, 1 ml. 0·37% takadiastase, 3 ml. citrate-phosphate buffer, pH 6·2, at 37°.)

Time (hr.)	Phenolphthalein liberated (μg.)	Phenolphthalein liberated (%)
4	23	0·73
	22	0·69
8	52	1·64
	51	1·60
16	96	3·02
	98	3·08
24	143	4·50
	140	4·40

Potassium 1-naphthylsulphate

Synthesis of potassium 1-naphthylsulphate. This compound was synthesized by the method of Feigenbaum & Neuberg (1941) using dimethylaniline as the organic base in the reaction. The potassium salt was purified by converting it to the *p*-toluidine salt by the procedure described by Barton & Young (1943), recrystallizing this compound, and then converting it back to the potassium salt. The product gave negative tests for free naphthol (see below) and inorganic sulphate. It gave positive tests for naphthol and inorganic sulphate after it had been heated with N-HCl in a boiling-water bath for 1 min. (Found: total sulphate S, 12·4. Calc. for $C_{10}H_7O_4SK$, 12·2%.)

Detection of 1-naphthol liberated by hydrolysis of potassium 1-naphthylsulphate. 1-Naphthol was detected by the red colour it gives in alkaline solution with diazotized sulphanilic acid. The test was carried out by making the solution alkaline with NaOH solution and then adding freshly prepared diazo reagent (1 vol. of 0·8% $NaNO_2$ solution mixed with 9 vol. of a 0·25% solution of sulphanilic acid in N-HCl).

Stability of the substrates under conditions used for bacteriological tests

In the present investigation experiments were carried out in which bacteria were allowed to grow at 37° for 7 days in media containing potassium

phenolphthalein disulphate or potassium 1-naphthylsulphate. In the tests with *Actinomyces* the period of growth was 14 days. It was necessary, therefore, to determine whether any breakdown of the substrates took place under these conditions apart from that produced by the bacteria.

Into each of a series of glass-stoppered test tubes were placed 3 ml. citrate-phosphate buffer, pH 6·0, 7·0 or 8·0, 1 ml. of 0·01M-potassium phenolphthalein disulphate or 0·01M-potassium 1-naphthylsulphate, and 1 ml. of 0·1% 'Merthiolate' solution (Eli Lilly and Co. Ltd.) as a preservative. The tubes were placed in an incubator at 37° and at intervals up to 28 days, tubes were removed and their contents tested for the presence of phenolphthalein or 1-naphthol. No evidence of the breakdown of the substrates was obtained even after 28 days, i.e. after a period twice as long as the longest period used in the bacteriological tests.

As the media to which the substrates were added were sterilized by autoclaving at 15 lb./sq.in. pressure for 20 min., it was also necessary to determine whether any liberation of phenolphthalein or 1-naphthol occurred during this process. Tests on media with pH 7·0–7·6 containing 0·001 M-potassium phenolphthalein disulphate or 0·001 M-potassium 1-naphthylsulphate which had been sterilized in this way gave no evidence that hydrolysis of the substrates had taken place. Furthermore, there was no indication of hydrolysis after 2–3 months storage of the sterilized media at 4°. No detectable loss of the substrates occurred when their solutions were sterilized by Seitz filtration.

THE DISTRIBUTION OF BACTERIAL ARYLSULPHATASE

Apart from the investigations of Barber *et al.* (1951) on staphylococci, information appears to be lacking concerning the distribution of bacterial arylsulphatase. In the present work, various species of bacteria of medical interest have been examined for the occurrence of this enzyme. In order to test for the presence of the enzyme, the organisms were grown in a medium containing potassium phenolphthalein disulphate or potassium 1-naphthylsulphate, and the appearance of free phenolphthalein or 1-naphthol in the medium was taken as evidence of the production of arylsulphatase by the organism.

Methods

Preparation of media. An 0·01 M solution of the substrate (potassium phenolphthalein disulphate or potassium 1-naphthylsulphate) in water was added to broth (pH 7·4–7·6) so as to produce a medium containing 1% meat extract (Lab Lemco, Oxo Ltd.), 1% peptone (Bacteriological Peptone, Evans Medical Supplies Ltd.), 0·5% NaCl, and 0·001 M-substrate. The medium was distributed in 4 ml. portions in $6 \times \frac{5}{16}$ in. tubes and autoclaved for 20 min. at 15 lb./sq.in. pressure.

For the cultivation of anaerobes, hot sterile vaseline was added to the tubes immediately after their removal from the autoclave. When cool, the medium was inoculated in the usual manner. The vaseline seal was first melted by gentle heat and, after the medium had been inoculated, the vaseline was allowed to re-seal the tube.

For certain species, where it was needed for growth, 0·5 ml. sterile horse serum or blood was added to each tube after autoclaving, the tubes containing blood being brought to 100° for 2–3 min. and well shaken. In some experiments the medium was enriched with 0·5% glucose.

Detection of arylsulphatase production by organisms grown in media containing phenolphthalein disulphate. The strain to be tested was inoculated into three tubes of broth containing 0·001 M-phenolphthalein disulphate, which were then incubated at 37°. One tube was tested for the presence of free phenolphthalein after 1 day, another after 3 days and the third after 7 days. Free phenolphthalein was detected by the addition of N-NaOH until maximum development of colour had taken place. Decolorization of phenolphthalein takes place in strongly alkaline solutions, and the NaOH solution was therefore added carefully drop by drop with shaking of the contents of the tube. The depth of colour which developed was assessed visually and recorded according to the scale shown in Table 2.

Detection of arylsulphatase production by organisms grown in media containing potassium 1-naphthylsulphate. Each strain examined with this substrate was inoculated into three tubes of broth containing 0·001 M-potassium 1-naphthylsulphate and also into three tubes of broth without substrate. Control experiments using broth without substrate were carried out because it was found that faintly positive diazo reactions were obtained after growth had occurred. After incubation at 37° for 1 day a tube containing substrate and a control tube were removed and tested by means of the diazo reaction. The remaining tubes were tested after incubation for 3 and 7 days. The diazo test was carried out by adding to each tube 1 ml. of 2 N-NaOH and 0·5 ml. of freshly prepared diazo reagent (1 ml. 0·8% $NaNO_2$ and 9 ml. 0·25% sulphanilic acid dissolved in N-HCl). The tubes were shaken and the difference in depth of colour between the tube containing naphthylsulphate and the control tube was assessed visually and recorded according to the scale shown in Table 2.

Sources of the strains studied. The strains examined were obtained from the following sources: the Stock Collection, Department of Bacteriology, St Thomas's Hospital Medical School; the Stock Collection and routine cultures, Louis Jenner Laboratory, St Thomas's Hospital; the Salmonella Reference Laboratory, Public Health Laboratory Service, Colindale; the National Collection of Type Cultures, Colindale. A collection of strains from cases of infantile gastro-enteritis was obtained from Dr G. S. Udall, St Thomas's Hospital Medical School.

The majority of the strains of salmonellae were inoculated directly from Dorset's egg medium. Strains of haemolytic streptococci, clostridia and corynebacteria were subcultured from Robertson's meat medium. The remainder were inoculated from nutrient or blood agar.

Results

Tests with potassium phenolphthalein disulphate as substrate. The number of strains tested with phenolphthalein disulphate as substrate was 212, and of these, fifty-four were found to show varying degrees of arylsulphatase activity. Among the organisms tested, the enzyme was found to be present in certain species of salmonellae and mycobacteria. For this reason the salmonellae and related bacteria

were studied more extensively than the other groups. The examination of large numbers of mycobacteria, many of which require special methods for their cultivation, was considered to be outside the scope of the present work and it is being made part of a separate investigation (Whitehead, Wildy & Engbaek, to be published).

Among seventy-nine strains of salmonellae, forty-seven strains belonging to fourteen species were found to give positive tests for the presence of arylsulphatase. No positive strains could be detected among nine strains of *Salm. typhosa*. Of the paratyphoid bacteria, all of the eleven strains of *Salm. paratyphi* tested, eight of twelve strains of *Salm. schottmuelleri*, and one of ten strains of *Salm. hirschfeldii* were found to give strongly positive tests. The strains of *Salm. paratyphi* showed somewhat weaker activity than the arylsulphatase-positive strains of the other paratyphoid bacteria. The four negative strains of *Salm. schottmuelleri* belonged to phage Types 1 and 2, and six of the positive strains to phage Types 3*a*, 3*a*1 and 3*b*. Unlike the paratyphoid organisms, the twenty-seven positive strains found among thirty-seven strains of other species of salmonellae showed weak arylsulphatase activity. Among the related bacteria, no activity was detectable among twenty strains of *Shigella*, twenty-nine strains of *Escherichia*, *Aerobacter* and *Klebsiella*, and twenty-one strains of *Proteus*.

The ten strains of mycobacteria examined included four strains originally isolated from cold-blooded animals. Of these four strains, two of *Mycobact. piscium* and one of *Mycobact. ranae* showed considerable activity, while a strain of *Mycobact. chelonei* was less active. Of the saprophytic acid-fast bacteria, two strains each of *Mycobact. phlei*, *Mycobact. smegmatis* and *Mycobact. stercoris* were tested. One strain of each species gave negative tests for arylsulphatase, and one strain of each species showed weak activity.

The following organisms, in addition to those already mentioned, gave no evidence of the production of arylsulphatase (where more than one strain was examined the number is shown in brackets): *Actinomyces* (anaerobic strain from human actinomycosis); *Bacillus anthracis*, *B. subtilis* (3), other Gram-positive sporing bacilli (5); *Clostridium perfringens*, *Cl. septicum*, *Cl. sporogenes*, *Cl. tetani*, *Cl. tetanomorphum*; *Corynebacterium diphtheriae* var. *gravis*, *C. diphtheriae* var. *intermedius*, *C. diphtheriae* var. *mitis* (3), *C. pseudodiphtheriticum*, *C. xerose*; *Diplococcus pneumoniae*; *Gaffkya tetragena*; *Haemophilus influenzae*, *Haem. pertussis*; *Micrococcus lysodeikticus*; *Neisseria meningitidis* (2); *Pasteurella pestis*; *Pseudomonas aeruginosa* (4); *Sarcina*; *Serratia marcescens*; *Streptococcus faecalis* (2), haemolytic streptococci (7), non-haemolytic streptococci (3), *Strep. viridans* (3); *Vibrio comma* (2).

Tests with potassium 1-naphthylsulphate as substrate. A number of tests for arylsulphatase were carried out using potassium 1-naphthylsulphate as substrate in order to find whether the results obtained would be similar to those from experiments in which phenolphthalein disulphate was used as substrate. Included in these tests were the majority of the salmonella strains which were negative or weakly positive, and a few which were strongly positive when tested on a substrate of phenolphthalein disulphate. In all, fifty-seven strains of this group were tested with a substrate of potassium 1-naphththylsulphate. The results are included in Table 2, and they show that there is a broad measure of agreement in the results obtained with the two compounds, although in some instances hydrolysis of the substrate was detectable earlier when potassium 1-naphthylsulphate was used. In addition to the salmonella strains, the following sixteen strains from other genera were tested with potassium 1-naphthylsulphate as substrate and they yielded results in accordance with those obtained with phenolphthalein disulphate: *Aerobacter aerogenes*, *B. anthracis*, *B. subtilis*, *Esch. coli* (2), *Klebsiella pneumoniae*, *Micrococcus epidermidis*, *M. pyogenes* var. *aureus*, *Mycobact. piscium*, *Past. pestis*, *Ps. aeruginosa*, *Proteus vulgaris* (2), *Shigella sonnei*, *Strep. pyogenes*, *V. comma*.

During the examination of some strains in media containing potassium 1-naphthylsulphate it was observed that after incubation for 24 hr. or more there was a partial clearing of the turbidity of the cultures. It was noticed that this only occurred with strains which produced arylsulphatase and never with those which did not.

Experiments using a colourless medium. In an attempt to detect slight degrees of hydrolysis of phenolphthalein disulphate by bacteria, trials were made with this compound in a colourless medium consisting of 1% peptone (Bacteriological Peptone, Evans Medical Supplies Ltd.) and 0·5% sodium chloride in water. The substrate concentration was 0·001M. This medium was used for the examination of sixty-seven strains from various genera tested using the other medium. It was found that although slight hydrolysis of phenolphthalein disulphate could more easily be detected in the colourless medium, this advantage was outweighed by the poorer growth which occurred with many organisms. No additional arylsulphatase-producing strains were detected with the colourless medium.

Experiments with a medium containing 0·5% glucose. In order to determine whether the production of arylsulphatase is affected by the enhanced growth which many bacterial species show in media containing glucose, two strains known to

produce the enzyme were grown in nutrient broth containing 0·001 M-phenolphthalein disulphate and, in addition, 0·5 % glucose. No arylsulphatase activity could be detected after incubation for 7 days, although the organisms showed more vigorous growth than was observed in the absence of glucose. All the salmonellae which had been shown to produce the enzyme were therefore examined in broth containing 0·5 % glucose and the substrate, and none showed any trace of arylsulphatase activity. Under the same conditions no activity could be detected in any of six strains of *Mycobact. phlei*, *Mycobact. smegmatis* and *Mycobact. stercoris*, whereas the four strains of *Mycobact. piscium*, *Mycobact. ranae* and *Mycobact. chelonei* showed no impairment of arylsulphatase activity when grown in broth containing 0·5 % glucose. Twenty strains from various genera which were

Table 2. *Results of testing seventy-nine strains of salmonellae for arylsulphatase activity using potassium phenolphthalein disulphate (I) and potassium 1-naphthylsulphate (II) as substrates*

(Substrate I. Colour on addition of alkali to the medium: ±, faint tinge of pink; +, pale pink; + +, pink; + + +, red; + + + +, deep red; −, no change in colour. Substrate II. Colour difference between control and medium containing substrate on addition of alkali and diazo reagent: ±, just discernible; + +, moderate; + + + +, marked; −, no difference. NT = strain not tested.)

Species of Salmonella	No. of strains tested	Arylsulphatase activity: 1 day, Substrate I	1 day, Substrate II	3 days, Substrate I	3 days, Substrate II	7 days, Substrate I	7 days, Substrate II
Salm. typhosa	9	−	−	−	−	−	−
Salm. paratyphi	1	−	+ +	+ +	+ + + +	+ + + +	+ + + +
	1	−	+ +	+ +	+ +	+ + + +	+ + + +
	3	±	NT	+ +	NT	+ + + +	NT
	4	±	NT	+ + +	NT	+ + + +	NT
	1	+	NT	+ + + +	NT	+ + + +	NT
	1	+ +	NT	+ + + +	NT	+ + + +	NT
	2	+ +	NT	+ + +	NT	+ + + +	NT
Salm. schottmuelleri Types 1 and 2	2	−	−	−	−	−	−
Salm. schottmuelleri Types 1 and 2	2	−	−	−	±	−	−
Salm. schottmuelleri Types 3*a*, 3*a*1, 3*b*	5	+ +	NT	+ + + +	NT	+ + + +	NT
Salm. schottmuelleri Type 3*b*	1	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + + +
Salm. hirschfeldii	1	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Salm. hirschfeldii	9	−	−	−	−	−	−
Salm. sp. (Type Aberdeen)	1	−	−	±	−	+	±
Salm. abortivoequina	1	−	−	−	−	−	−
Salm. morbificans	1	−	−	−	−	±	−
Salm. choleraesuis	1	−	−	−	−	−	−
Salm. sp.	1	−	−	−	±	±	+ +
Salm. sp. (Type Dublin—human)	1	−	±	−	±	+	+ +
Salm. sp.	1	−	−	−	−	−	±
Salm. sp.	1	−	−	−	−	+ +	+ +
Salm. sp. (Type Dublin—bovine)	1	−	−	−	−	±	±
Salm. sp.	1	−	−	+ +	±	+ +	+ +
Salm. enteritidis	1	−	NT	−	NT	±	NT
Salm. enteritidis	2	−	−	−	−	±	−
Salm. enteritidis	1	−	−	−	−	±	±
Salm. enteritidis	1	−	−	+ +	−	+	−
Salm. sp. (Type London)	1	−	−	−	±	±	−
Salm. sp. (Type Newington)	1	−	−	−	−	±	±
Salm. sp. (Type Newington)	1	−	−	±	−	+ +	±
Salm. sp. (Type Newport)	1	−	NT	±	NT	+ +	NT
Salm. sp. (Type Newport)	2	−	−	−	−	±	±
Salm. sp. (Type Newport)	1	−	−	−	−	±	−
Salm. sp. (Type Poona)	1	−	−	−	−	+	−
Salm. sp. (Type Potsdam)	1	−	−	−	−	±	−
Salm. sp. (Type Senftenberg)	1	−	−	−	−	−	−
Salm. sp. (Type Thompson)	1	−	−	±	−	+ +	±
Salm. sp. (Type Thompson)	1	−	NT	−	NT	+	NT
Salm. sp. (Type Thompson)	1	−	−	−	−	−	−
Salm. sp. (Type Thompson)	2	−	−	−	−	−	±
Salm. sp. (Type Thompson)	1	−	−	−	−	−	+ +
Salm. typhimurium	1	−	NT	−	NT	−	NT
Salm. typhimurium	1	−	−	−	−	−	−
Salm. typhimurium	2	−	−	−	−	±	±
Salm. typhimurium	2	−	−	−	−	±	−

negative when tested in nutrient broth showed no arylsulphatase activity when grown in glucose broth.

Experiments under anaerobic conditions. Two strains of *Salm. schottmuelleri* and one of *Salm. paratyphi* which produced arylsulphatase when grown aerobically were cultured under anaerobic conditions by the methods described earlier. In each case visible growth was less than when these organisms were grown aerobically, but arylsulphatase production appeared to be only slightly diminished.

SEPARATION AND PROPERTIES OF BACTERIAL ARYLSULPHATASE

In order to study the properties of bacterial arylsulphatase, experiments designed to separate the enzyme from bacteria were undertaken. As a result of this work it was found possible to prepare cell-free aqueous solutions of arylsulphatase and these have been used for preliminary studies of the properties of the enzyme.

The organism from which arylsulphatase was separated was a subculture of *Mycobact. piscium*, N.C.T.C. No. 2291. This was obtained originally about 10 years ago from the National Collection of Type Cultures, and since then the strain has been maintained by monthly subculture on nutrient agar slopes. The original strain was isolated from the diseased roe of a halibut by Griffith (1930). This organism was selected for the present investigation because its arylsulphatase activity was high compared with that of other organisms which had been tested. Additional advantages attending its use were its lack of pathogenicity to man and the fact that its form of growth on a liquid medium is such that it can be harvested easily. At 37° growth of the organism reaches a maximum after about 5–7 days and on liquid media it takes the form of a moderately tenacious yellowish grey pellicle with little subjacent turbidity or deposit, thus rendering harvesting by filtration a simple procedure.

Growth and harvesting of the bacteria. The organism was grown in 4 oz. 'medical flat' bottles loosely plugged with cotton wool, each containing 30 ml. nutrient broth containing 0·001 M-potassium phenolphthalein disulphate. Inoculation was carried out by carefully floating on to the surface of the medium a portion of surface pellicle (approx. 1·5 cm. in diameter) from a 3–5-day broth culture. The bottles were then incubated in a horizontal position so as to expose the greatest surface area of medium for growth of the organisms. The growth was harvested after 5–7 days incubation at 37°, by which time the surface of the medium was almost completely covered by the pellicle. The bacterial mass was separated by filtration on a Büchner funnel using a no. 1 Whatman paper and it was washed with about 100 ml. of distilled water for each bottle whose contents were filtered. Most of the excess moisture was removed by suction on the filter.

Extraction of the enzyme from the bacterial mass. Examination of the culture filtrate showed that its arylsulphatase activity was low. Attempts were made, therefore, to obtain active preparations of the enzyme by extracting the bacilli. No arylsulphatase activity was found in extracts obtained by hand-grinding moist bacilli or acetone-dried bacilli in an agate mortar with saline-phosphate buffer at pH 7·0. In each case tests showed that the activity remained in the residue of the ground bacilli. Active preparations were obtained, however, when the technique of mechanical grinding of bacteria recommended by Dockstader & Halvorson (1950) was employed. The procedure which was developed for the preparation of cell-free aqueous solutions of the enzyme was as follows.

The bacterial mass which had been separated by filtration on a Büchner filter and washed with distilled water was dried by repeated washing with acetone followed by suction. The dried material was scraped off the filter paper and weighed. It was then mixed with twice its weight of Hyflo Super-Cel (Johns-Manville Co. Ltd.) which had previously been washed three times with distilled water and dried. The mixture was placed in a homogenizer (Potter, 1945) and twice its volume of distilled water was added. The tube containing the mixture was immersed in an ice bath for 15 min. while its contents were ground at about 1500 rev./min. The viscid creamy material obtained by grinding was suspended in ice-cold distilled water (1 ml./5 mg. of acetone-dried bacilli) and centrifuged at 1800 *g* for 30 min. The opalescent supernatant liquid was decanted and filtered through a Gradocol collodion membrane (average pore diameter, 0·52 μ.) in an Elford ultrafilter to remove any remaining bacterial cells. The filtrate obtained was clear and showed only a very faint yellowish tinge. Dry weight determinations on two filtrates obtained in this way gave values of 1·42 and 1·57 mg./ml.

By means of the procedure just described it was possible to obtain cell-free solutions of arylsulphatase from bacilli grown in the presence of phenolphthalein disulphate. Even when the procedure was modified by the omission of phenolphthalein disulphate from the medium the final solution obtained was found to contain the enzyme. Arylsulphatase was also separated from bacilli grown from an inoculum of organisms which had never been in contact with phenolphthalein disulphate.

Optimum pH *of the enzyme.* Experiments were conducted in which the enzyme was allowed to act on potassium phenolphthalein disulphate at various pH values ranging from 3·0 to 8·0. The experiments were carried out in glass-stoppered Pyrex test tubes into each of which was placed 3·0 ml. of citrate-phosphate buffer (McIlvaine series; Britton, 1942), 1·0 ml. of 0·01 M-potassium phenolphthalein disulphate and 1·0 ml. of unbuffered enzyme preparation. After 6 hr. in the water bath at 37° the tubes were transferred to an ice bath and the free phenolphthalein was determined by the method

described earlier in the present paper. The results obtained from duplicate experiments with each of two enzyme preparations are shown in Table 3. Experiments in which the pH values of the buffer-substrate-enzyme systems were determined electrometrically at the beginning and the end of the period of incubation revealed no significant change in pH. No detectable liberation of phenolphthalein occurred in 6 hr. at 37° in control experiments in which water was used in place of enzyme solution at each pH value studied, or in which enzyme solution which had been heated in a boiling-water bath for 20 min. was allowed to act on the substrate at pH 6·3.

Table 3. *Optimum* pH *of arylsulphatase present in cell-free extracts of a strain of* Mycobacterium piscium

(3·0 ml. citrate-phosphate buffer, 1·0 ml. 0·01 M-potassium phenolphthalein disulphate, and 1·0 ml. enzyme solution at 37° for 6 hr.)

pH	Phenolphthalein liberated (μg.) Enzyme preparation *A*	Enzyme preparation *B*
3·0	2	—
4·0	12	—
5·0	50	—
5·6	—	21
6·0	59	23
6·3	—	24
6·7	—	20
7·0	47	16
8·0	9	—

Table 4. *Optimum substrate concentration of arylsulphatase present in cell-free extracts of a strain of* Mycobacterium piscium

(3·0 ml. citrate-phosphate buffer pH 6·3, 1·0 ml. potassium phenolphthalein disulphate solution, and 1·0 ml. enzyme solution at 37° for 6 hr.)

Substrate concn. in system (M)	Phenolphthalein liberated (μg.)
0·0005	13
0·0010	19
0·0020	21
0·0030	21
0·0040	20
0·0050	20

From the results given in Table 3 it appears that under the conditions employed the optimum pH of the arylsulphatase present in the extracts lay in the vicinity of 6·3.

Optimum substrate concentration. In order to determine the optimum substrate concentration a series of experiments was carried out in each of which 3·0 ml. of citrate-phosphate buffer pH 6·3 and 1·0 ml. of enzyme solution were heated for 6 hr. at 37° with 1·0 ml. of potassium phenolphthalein disulphate solution and the phenolphthalein liberated was determined colorimetrically by the method already described. The phenolphthalein disulphate solutions used were such that the concentrations of substrate in the system ranged from 0·0005 to 0·005 M at the beginning of the experiment. The results obtained are shown in Table 4 from which it appears that the maximum velocity of arylsulphatase action occurred with a substrate concentration of 0·002–0·003 M.

DISCUSSION

As the present investigation involved the testing of many different bacteria for the presence of arylsulphatase, it was necessary first to devise a simple, sensitive test for the enzyme which could be applied in a medium suitable for the growth of a wide variety of organisms. Various substrates have been used for the qualitative and quantitative study of plant and animal arylsulphatase. These include potassium indoxyl sulphate (Derrien, 1911; Neuberg & Wagner, 1925), potassium phenylsulphate (Neuberg & Kurono, 1923; Abbott, 1947), and potassium 4-nitrophenylsulphate (Neuberg & Wagner, 1925; Huggins & Smith, 1947), and while the present work was in progress, Robinson, Smith & Williams (1951) described the use of potassium 2-hydroxy-4-nitrophenylsulphate for the study of the enzyme. The use of these compounds has been based on the fact that on hydrolysis they yield phenolic products which can be detected or determined by their conversion to coloured compounds on treatment with suitable reagents. In applying this principle to the detection of bacterial arylsulphatase, consideration has to be given to the fact that culture media often have a yellow colour, and in consequence compounds which on hydrolysis yield phenols which are detected by their conversion to yellow products (e.g. potassium 4-nitrophenylsulphate) are unsatisfactory. In order to study bacterial phosphatase, Bray & King (1942) used phthalein phosphates as substrates and later they made a study of the distribution of phosphatase in micro-organisms using phenolphthalein diphosphate as substrate (Bray & King, 1943). Phenolphthalein offers a number of advantages as a chromogen, the chief of which are that the red-coloured form can be obtained merely by the addition of alkali, very small amounts of phenolphthalein are readily detected, and the colour reaction is not susceptible to interference by substances present in biological material. For these reasons phenolphthalein diphosphate has been used for work on phosphatases (Bray & King, 1943; Huggins & Talalay, 1945; Barber *et al.* 1951) and phenolphthalein glucuronide has been used for the

study of β-glucuronidase in mammalian tissues (Talalay *et al.* 1946) and in micro-organisms (Barber *et al.* 1951). These various considerations led us to use phenolphthalein disulphate as an agent for testing for arylsulphatase. Some tests were also carried out with a monosulphate, potassium 1-naphthylsulphate, as substrate, in order to determine whether the use of a different substrate would influence our conclusions concerning the distribution of arylsulphatase. The results obtained with this compound, however, were in general agreement with those obtained using phenolphthalein disulphate. As an agent for testing for arylsulphatase in bacteria, potassium 1-naphthylsulphate suffers from the disadvantage that it is necessary to run control experiments, for most organisms yield faintly positive diazo reactions after growth has occurred in the absence of the substrate.

The organisms tested for arylsulphatase activity in the present work did not include staphylococci or anaerobic cocci. The distribution of arylsulphatase among staphylococci has been investigated by Barber *et al.* (1951) and the distribution of the enzyme among anaerobic cocci has been studied in the course of an investigation by Prof. R. Hare & Dr M. P. L. Wildy of the Bacteriology Department of this School (to be published). A wide variety of organisms was examined in the present work, and the results suggest that the possession of arylsulphatase in readily detectable amounts is not common among bacteria. Arylsulphatase activity was found in certain species of salmonella and mycobacteria, but with the methods used it could not be detected in any of the other species tested. The possibility should not be overlooked that the examination of larger numbers of strains of a particular species might reveal the occurrence of further arylsulphatase-producing organisms. It must also be recognized that, whereas a positive finding is significant in an investigation of this type, a negative finding does not necessarily exclude the possibility that the organism produces the enzyme. Variation of the cultural conditions or the use of a more sensitive method of testing might reveal the presence of the enzyme in an organism with weak arylsulphatase activity. In this connexion it should be noted that tests carried out on organisms of the Salmonella group with a monosulphate, potassium 1-naphthylsulphate, revealed the occurrence of weak arylsulphatase activity in a few strains earlier than when phenolphthalein disulphate was used as the substrate, although in most cases the two tests were in agreement. Another possibility which has to be considered is that the phenolic compound liberated from the substrate by the action of the enzyme might be metabolized by the organism and thus escape detection. This question is under investigation by Whitehead, Wildy & Engbaek (to be published) in their study of the arylsulphatase activity of mycobacteria using phenolphthalein disulphate as substrate, but no evidence has so far been obtained suggesting that utilization of phenolphthalein by these organisms (whether they produce arylsulphatase or not) occurs to an extent likely to affect the detection of the enzyme. Whether this is generally true of other bacterial species has not been established.

From a biochemical standpoint considerable interest attaches to the separation of the enzyme and the study of its properties. Among the various bacteria studied, one of the most active producers of arylsulphatase was a strain of *Mycobact. piscium*. Examination of the culture medium of this organism grown in the presence or the absence of phenolphthalein disulphate suggested that the medium contained little or no arylsulphatase. When the organism itself was separated and dried with acetone, it was found possible to prepare cell-free aqueous extracts which contained much of the arylsulphatase activity originally present in the dried organisms. These extracts were used for studies of the optimum pH and optimum substrate concentration of the enzyme. These preliminary experiments on the separation and properties of bacterial arylsulphatase have paved the way for more extensive biochemical studies of the enzyme.

SUMMARY

1. A description is given of the synthesis and properties of potassium phenolphthalein disulphate.

2. Potassium phenolphthalein disulphate has been used as a substrate for testing for the presence of arylsulphatase (phenolsulphatase) in 212 strains from a wide range of bacterial species, and seventy-three of these strains have also been tested for the presence of this enzyme using potassium 1-naphthylsulphate as substrate. The results obtained with the two substrates are in general agreement.

3. Under the test conditions, arylsulphatase has been detected only in certain strains of salmonellae and mycobacteria.

4. Cell-free aqueous extracts containing arylsulphatase have been prepared from *Mycobacterium piscium*, N.C.T.C. 2291, and these have been used in a preliminary study of the properties of the enzyme.

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Microdetermination of Manganese in Biological Material by a Modified Catalytic Method

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In investigating the biochemical roles of manganese it is desirable to employ a very sensitive but reasonably accurate method of estimating the element. Methods based on the detection of manganese lines in emission spectra require the presence of about 50 μmg. of the element in a 10 mg. sample, and even when determinations are carried out in triplicate the accuracy obtainable is not great.

The periodate method, based on oxidation of the manganese to permanganate, which is determined by photoelectric colorimetry or spectrophotometry (Willard & Greathouse, 1917; Richards, 1930; Strickland & Spicer, 1949; Davidson & Capen, 1929; Mehlig, 1939, 1941; Kirk, Rosenfels & Hanahan, 1947; Rowland, 1939) is generally satisfactory. The main disadvantage of the periodate method is that the amount of tissue needed to provide the necessary minimum of manganese is quite often larger than that available. The colorimetric estimation of manganese by means of the Folin-Ciocalteu reagent (Abul-Fadl, 1948) requires at least 12 μg. manganese.

Szebelledy & Bartfai (1936) described a catalytic method for the detection of manganese in amounts less than 1 μmg., and Kun (1947) worked out a quantitative procedure, using photoelectric spectrophotometry. Manganous ions catalyse the oxidation of *NN*-diethylaniline by potassium metaperiodate (KIO_4) in aqueous solution, and the progress of the oxidation can be followed by measuring the increasing absorption of light at 470 mμ. Kun used a medium buffered at pH 7·0 and measured the extinction exactly 200 sec. after mixing the reagents. He found no interference by ions of other metals. Each test required at least 4 μmg. of manganese, but Kun found it necessary to make many readings so as to eliminate the effect of what appeared to be random variations.

The catalytic method appeared to be very promising, but a considerable study of all the variables was necessary before it could be applied with confidence to the problems of determining manganese in very small amounts of tissues. The final procedure adopted will first be described and then discussed.

EXPERIMENTAL

Reagents

All reagents must be stored in Pyrex vessels.

Glass-distilled water. Distilled water from the usual type of laboratory still is redistilled from all-glass Pyrex apparatus.

Hydrochloric acid. Spray-free hydrogen chloride is passed, via a wax-coated funnel, into glass-distilled water in a wax-lined beaker immersed in ice. The acid (approx. 16% w/v) is stored in wax-lined bottles.

Buffer solution (pH 7·0). This is prepared by mixing solution *a* (40 ml.) and solution *b* (approx. 30 ml.) and making up to 100 ml. Solution *a* consists of citric acid (A.R., 111 g.) and 'pure' conc. HCl (29 ml.) in 2·5 l. Solution *b* is prepared as follows: approx. 2N-NaOH (A.R. carbonate-free) is standardized and boric acid (A.R., 122 g.) is dissolved in exactly 2·5 equiv. of the alkali solution and the whole made up to 2·5 l.

Diethylaniline hydrochloride solution. Diethylaniline (0·12 g.) is weighed accurately and dissolved in 1·4 equiv. of 0·1N-HCl. The solution is made up to 1 l. (0·0008M).

Potassium periodate solution. KIO_4 solution (0·01M) is made by dissolving 2·3 g. of the A.R. salt in 800 ml. of hot water, cooling and diluting to 1 l. in a graduated flask.

Manganese solution. A.R. $MnSO_4.4H_2O$ was used for preparing the standard manganese solution.

Method

A 'translucent Vitreosil' crucible is cleaned with abrasive soap (Briz), boiled in conc. HCl, washed in glass-distilled water and heated for 30 min. at 650°. It is cooled in a desiccator, then weighed and after inserting the fresh tissue sample, weighed again. The tissue is dried to constant weight at 110° and is then ashed at not over 650° in a silica-lined muffle furnace. Glass-distilled water may be added to the cooled residue to assist removal of the last traces of carbon (this step is empirical but effective). The crucible is cooled and weighed, platinum-tipped crucible tongs being used throughout.

A measured volume of the 16% HCl is added to the crucible; this is placed inside a 100 ml. beaker and left on a steam bath until the HCl has evaporated. Buffer solution (volume depending on the amount of Mn in the sample) is added to the crucible which is then placed on the steam bath for 20–30 min. (2 ml. of buffer is used for samples containing up to 50 μmg. including the Mn contaminating the HCl. With larger samples proportionately more buffer is added. When the amount of Mn is very large the crucible is extracted with a known volume of HCl instead of with buffer and the solution washed into a graduated flask of suitable size. After making up to volume, a portion of the solution is dispensed into a clean crucible, evaporated to dryness and then taken up in buffer as described above.) The beaker is then removed and allowed to cool, protected from dust. With the aid of a small Pyrex funnel and a wash-bottle (Pyrex) with the shortest possible rubber connexion, the contents of the crucible are washed into a graduated flask of a capacity 2·5 times the volume of buffer taken. Pyrex graduated flasks should be used if obtainable.

Four such buffered solutions of ash are made up, together with a blank consisting of diluted buffer alone and a standard consisting of diluted buffer containing 4·00 μmg. Mn/ml. These six solutions, the reagents, six 1 ml. pipettes and 36 Pyrex test tubes (9 × 75 mm. with rim), together with other necessary apparatus, are left in the instrument room overnight. Within 24 hr. of making the buffered ash solutions and at a time when the room temperature is fairly stable three 1 ml. portions of each buffered solution are dispensed into test tubes and 1 ml. portions of KIO_4 solution into the remaining 18 tubes. Taking the solutions in the order in which they were pipetted (three blanks, four × three buffered ash solutions, then three standards) 0·5 ml. of diethylaniline hydrochloride solution is added from a graduated pipette to the buffered solution. The mixture is immediately poured into the KIO_4 solution, the time at that instant noted on a stop-clock and the reaction mixture agitated by repeatedly pouring from one tube to the other. The solution, in which the colour is now developing, is transferred to a clean dry absorption cell (1·0 cm.) and placed in the photoelectric spectrophotometer. The *E* value of the solution is read exactly 60 sec. after mixing.

The cell containing the reaction mixture is rinsed out four to six times with glass-distilled water, three times with redistilled absolute ethanol and three times with redistilled ether. It is then allowed to dry with a current of dust-free air blowing through it and another cell, used in the previous measurement, is used for the next reaction mixture. At the end of the run the pipettes used for the six buffered solutions are washed out in the manner described for the cells. The test tubes are all cleaned carefully before use. New ones are first cleaned with abrasive soap, but the procedure used for cleaning the tubes for each run is as follows: the tubes are filled with HCl (1 vol. conc. acid + 1 vol. water) and allowed to stand for a few minutes. The acid is poured out and the tubes rinsed three times with tap water, three times with distilled water and three times with glass-distilled water. The tubes are then drained, dried in an electric oven and stored away from dust.

The mean of the three extinction values (*E*) for each buffered solution is obtained. The means for the blank and standard are used to fix the standard straight line from which the Mn concentrations corresponding to the *E* values for the four test solutions are interpolated. From the Mn concentration in the reaction mixture the amount of Mn derived from each sample can be calculated and hence the Mn concentrations in fresh tissue, dried tissue and ash.

RESULTS

The above procedure was developed as a result of an exhaustive investigation of the reaction conditions. The experimental findings can be summarized as follows:

(*a*) A minimum of 1 μmg. of manganese is required for a single determination, but owing to variability in the colour intensity produced, three determinations must be made for each sample examined. In practice the sample should contain at least 5 μmg. of manganese. With the reagents used in this method, the extreme sensitivity (to 0·1 μmg.) claimed by Szebelledy & Bartfai in 1936 for a catalytic method of detecting manganese was not attainable in our hands.

(*b*) The manganese concentrations in the reaction mixture itself could be determined to ± 10%

Table 1. *Comparison of the catalytic and periodate methods*

Wt. of liver taken (g.)	Sample taken	Estimated Mn (μg.)	Total Mn from liver (μg.)	Mn content (p.p.m.)
		Periodate method		
9·98	—	27·2	27·2	2·73
10·26	—	27·3	27·3	2·67
				Mean 2·70 ± 1 %
		Catalytic method		
10·24	1/100	0·303	30·3	2·96
	1/100	0·274	27·4	2·67
10·36	1/100	0·262	26·2	2·53
	1/100	0·265	26·5	2·56
				Mean 2·69 ± 10 %

(deviation from the mean) over the range 0·4–4·0 μmg./ml. and this permitted similar accuracy in determinations of the manganese contents of moderately large samples of tissue. With smaller samples (containing less than 10 μmg. Mn) reproducibility was not quite so good, possibly owing to contamination, and the accuracy was about ± 20 %. In Kun's experience the accuracy attainable for the determinations of manganese concentration over the range 0·4–40 μmg./ml. was ± 15 %.

(*c*) The rate of the reaction was directly proportional to the manganese concentration under the conditions already described. Kun obtained a logarithmic relationship but his experimental conditions differed considerably from ours.

(*d*) The possibility of interference was studied for the following ions: Cr^{+++}, Co^{++}, Cu^{++}, Fe^{++}, Ni^{++}, Mg^{++}, K^{+}, Hg^{++}, Sn^{++}, Pb^{++} and Zn^{++}. In each case phosphate ions were present in the buffer. The only effect noticed was that the first three ions exerted an inhibitory effect, but in no case was it shown at a concentration small enough to have relevance to the determinations on tissues.

(*e*) To obtain satisfactory results the pH must be kept constant to within 0·1 unit. The reagents need to be kept overnight in the instrument room and the actual determinations on the spectrophotometer must only be made when the room temperature is steady.

(*f*) By using diethylaniline hydrochloride solution containing excess hydrochloric acid, it was found that the reagent need not be freshly prepared, but should not be more than a fortnight old.

(*g*) It was important to adhere exactly to the procedure described for mixing the solutions, otherwise the intensities of absorption and the shape of the standard curve varied markedly.

(*h*) The addition of hydrochloric acid to the ash and subsequent evaporation to dryness is an essential step in ensuring that the ash is soluble in the buffer. It also serves to convert any Mn^{++++} or Mn^{+++} present in the ash to Mn^{++}. The hydrochloric acid contained a trace of manganese, even when prepared as described (p. 595), and allowance had to be made for this in working out the results.

(*i*) Excellent agreement was obtained when the manganese content of ox liver was determined by both the catalytic method and the periodate method using large samples. The results of such an experiment are given in Table 1.

Table 2. *Comparison of the catalytic and periodate methods using small samples for the former and silica or platinum crucibles for both*

Wt. of liver (g.)	Estimated Mn (μg.)	Mn content (p.p.m.)
Periodate method (silica crucibles)		
3·05	6·5	2·1
2·90	5·0	1·7
2·18	4·0	1·8
		Mean 1·9 ± 11 %
Periodate method (platinum crucibles)		
1·90	4·5	2·4
2·11	4·7	2·2
		Mean 2·3 ± 4 %
Catalytic method (silica crucibles)		
(mg.)	(μmg.)	
2·47	6·2	2·5*
1·24	5·1	4·1*
1·18	0·6	0·5*
1·23	1·8	1·5*
		Mean 2·2 ± 77 %*
Catalytic method (platinum crucibles)		
1·14	9·9	8·7
1·12	8·6	6·8
		Mean 7·8 ± 13 %

* These results show that the method is only semi-quantitative for amounts of material as small as about 1 mg.

(*j*) Samples ashed below 650° in silica crucibles lost some manganese by combination with the silica and were therefore expected to give low results. When very small samples for determination by the catalytic method were ashed in platinum

crucibles, however, the results obtained were higher than those obtained by the periodate method using larger samples also ashed in platinum crucibles. The results obtained by the catalytic method using very small samples ashed in silica crucibles agreed well with those (presumably the true ones) obtained by the periodate method using platinum crucibles. This was presumably due to a fortuitous compensation for the loss due to combination with the silica by some unknown factor. Table 2 illustrates this point with the results obtained when the method was applied to a protein powder prepared from ox liver.

(*m*) When the sample examined (e.g. blood) contained a large excess of iron the reaction mixture was coloured green and the readings obtained at 470 mμ. were below those for the blank. Thus determination of manganese in such material by the catalytic method was not possible.

Mechanism of the reaction

No direct attempt has been made to elucidate the mechanism of the catalytic process, but the development of the method yielded some relevant information.

Table 3. *Results of experiments with added manganese*

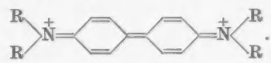
Sample of tissue (mg.)	Mn added (μmg.)	Mn estimated (μmg.)	Mn content (p.p.m.) Estimated	Calculated
54·2	Nil	11·7	0·22	
54·2	Nil	10·7	0·20	
			Mean **0·21**	
54·2	10·0	21·1	0·39	
54·2	10·0	25·2	0·46	
			Mean **0·43**	0·39
54·2	20·0	29·8	0·55	
54·2	20·0	36·2	0·67	
			Mean **0·64**	0·58
54·2	30·0	41·8	0·77	
54·2	30·0	38·6	0·71	
			Mean **0·74**	0·76

This table also shows that, for samples containing less than 5 μmg. of manganese, the deviation of the results from the mean is no longer within $\pm 20\%$. Kun observed good agreement between his catalytic method and a method based on the oxidation of manganese to MnO_4^-, but in making the comparison he used relatively large samples only.

(*k*) A puzzling phenomenon was observed in some calibration experiments. The usual procedure of adding known amounts of manganese to the ash of a sample and determining the manganese present was followed. The individual differences between calculated and observed manganese content bore no relation to the relative amount of added manganese and the observed values were, on the average, 10% higher. Table 3 gives the results of one such experiment in which eight samples of an acid solution of an ox eye-tissue (sclera) ash were evaporated in separate crucibles, known amounts of manganese being added to six of them. The manganese in all the crucibles was then determined.

(*l*) It was hoped that by omitting the phosphate constituent of Kun's buffer the catalytic method might be adapted to the determination of manganese in bone but interference by the large excesses of calcium and phosphate ions proved an insurmountable obstacle.

It appears that the potassium periodate oxidizes Mn^{++} to MnO_4^- probably by the mechanism studied by Strickland & Spicer in 1949. The MnO_4^- then oxidizes the diethylaniline to a 1:4-quinonoid compound, possibly a diquinone of the type



The manganese is then reoxidized to MnO_4^- by the periodate. Under conditions where there is excess of periodate and diethylaniline, as in the early stages of the reaction, a direct relationship between manganese concentration and rate of reaction would be expected when only a trace of the catalyst is present.

SUMMARY

1. A procedure for the determination of amounts of manganese of the order of 5 μmg. is described. Precise adherence to the instructions is essential for the success of the method.

2. The method cannot be applied to the determination of manganese in bone, nor, without further study, to manganese in blood.

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The Manganese in Bone

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Manganese is generally agreed to be essential for the prevention of perosis, a bone deformity of chicks and turkeys, and it may perhaps also be essential for normal bone formation in mammals. The mode of action of manganese in calcium and phosphorus metabolism has not been established but a role as activator of phosphatase has been suggested because chicks receiving added manganese show a rise in the manganese content of bone and an increase in phosphatase activity.

The present work is concerned not only with the manganese content of bone but also with the distribution of the element among its constituent parts, in the hope of finding new evidence pertinent to the elucidation of function.

EXPERIMENTAL

Analytical methods

Emission spectrography. A Littrow type quartz spectrograph (Hilger E_1) was used in two settings covering the regions 2260–2880 and 2870–5050 A. on 10 × 4 in. (25 × 10 cm.) plates. The light source was a d.c. arc (230 V.) between graphite or graphite-copper electrodes. For most of the work the copper-on-graphite electrodes were used; two 1 cm. lengths of pure copper rod (5 mm. diam.) were pushed into holes bored into the ends of graphite rods (9·5 mm. diam.). When the arc was running the electrodes became much hotter than simple copper electrodes and trace metals present in ash placed on the lower electrode were well volatilized into the flame. The *raies ultimes* of Mn (4030·8, 4033·1, 4034·5 A.) are easily obscured by cyanogen emission bands when graphite electrodes are used and are only shown with the copper-on-graphite arc (5 amp., 3 min. exposure) when the Mn concentration in bone ash is between 1 and 10 p.p.m. The 2800 A. triplet (2794·8, 2798·3 and 2801·1 A.) was more readily recorded than the Mn II *raies ultimes* (2576·1, 2593·7 and 2605·7 A.) and could be seen using graphite or copper on graphite electrodes at 1–10 p.p.m. Mn. Calcium phosphate ($Ca_3(PO_4)_2$) prepared from Analar Na_2HPO_4 and $CaCl_2$ revealed added Mn at 5 and 10 p.p.m. clearly, 2 and 4 p.p.m. with less regularity and certainty, and failed to show up 1 p.p.m.

Periodate method. The method of Skinner & Peterson (1930) was used in a modified form ensuring complete solution of the sample in H_3PO_4.

Materials and procedures

Fresh ox femurs from the abattoir were used.

(i) After removing flesh and marrow, the bone was cut into sections which were then reduced to shavings on a lathe, taking care to avoid contamination. The shavings, consisting of both compact and cancellous bone, were subjected to extraction with glass-distilled water saturated with $CHCl_3$. Three fractions were obtained, an aqueous phase, the residual shavings, and fat removed by means of ether from the extract. The bone marrow was shaken with ether for 4 hr. and on standing aqueous and ethereal phases separated, with gelatinous material at the interface. Much of this material appeared to consist of cell-wall debris from the marrow tissue, and was subsequently separated and washed with glass-distilled water. The two aqueous extracts were separately evaporated to dryness and the residues incinerated. The two ethereal extracts were washed with water, dried over Na_2SO_4, and the ether removed from measured portions and the residues burned off. The shavings and the 'cell-wall debris' were each dried and ashed. The six samples of ash were examined spectrographically on graphite and on copper-on-graphite electrodes.

(ii) (*a*) Fresh bone shavings were decalcified as follows: shavings (20 g.) were shaken with 0·1N-HNO_3 (1 l.) made from redistilled acid diluted with glass-distilled water. The suspension was filtered and the filtrate and residue subjected to emission spectrum analysis.

(*b*) The above treatment was repeated on a fresh sample (20 g.) and the residual shavings were left to stand overnight in a further 0·5 l. of 0·1N-HNO_3. The second extract and the (largely organic) residue were examined spectrographically. The very small amount of ash from the decalcified material was diluted with 'specpure' $(NH_4)_2SO_4$.

(iii) Two types of shavings were obtained from an ox femur, namely compact bone from the shaft and cancellous bone tissue from the head of the femur. Aqueous extracts from each type of material were made and portions were incubated with disodium phenylphosphate at pH 9 with suitable controls. After 16 hr. the solutions were tested for free phenol using 2:6-dichloroquinone chloroimide. The extracts were also tested spectrographically for Mn.

(iv) Another fresh ox femur was cut into sections and the marrow removed as completely as possible. The different portions of bone consisted of (*a*) predominantly epiphyseal bone from the head, (*b*) a mixture of epiphyseal and diaphyseal bone, and (*c*) diaphyseal bone from the shaft.

The separate portions of bone were incinerated and the proportions of ash determined.

(v) A portion of bone shaft was cleaned and turned on the lathe. Three experiments were carried out on the shavings. (*a*) 30 g. were packed in a 'chromatography' tube and covered with 0·1N-citric acid solution (made up in glass-distilled water). A dropping funnel was affixed to the top by means of a rubber bung and 3 l. of citric acid solution were allowed to percolate slowly through the shavings. Finally the contents of the tube were removed, dried and incinerated; the percolate was also dried and reduced to ash. The ash from the partly decalcified shavings was examined spectrographically and the ash from the percolate was analysed for Mn by the periodate method. (*b*) 16·6 g. of shavings were treated similarly and 9·3 g. of ash were obtained from the percolate and 0·31 g. from the residue in the tube. Both fractions were tested for Mn. (*c*) 30 g. of shavings were treated as before and the percolate collected in five fractions which were dried and incinerated separately. The residue in the tube consisted of an upper translucent portion and a lower opaque portion; the two portions were separated and each reduced to ash and examined for Mn.

RESULTS

Exp. (i) showed that the water-extracted shavings contained manganese (< 5 p.p.m.), but the ash from soluble material certainly contained no more. The aqueous extract from the bone marrow did not contain detectable amounts of manganese, but the ash from the cell-wall debris was richer than the total ash; it was enriched in manganese (10 p.p.m.) and in all other metals detected in bone (Al, B, Ba, Cu, Fe, Mg, Na, Pb, Sn, Sr and Zn) except potassium. Manganese was also detectable in the ash from both fatty fractions. The experiment showed that only a minute fraction of the total manganese in bone was present in a water-soluble form, and on the assumption that the bone phosphatase is extractable, little if any manganese can be directly attached to it. The small amount of manganese accompanying the fat may have been attached to the phosphoric acid residues of phospholipins.

Exp. (ii) showed that the organic matter remaining after decalcification with nitric acid retained a small amount of inorganic matter which was richer (on ashing) in manganese, copper, and iron than the original ash from the untreated shavings. The Mn content was about 50 p.p.m.

Exp. (iii) showed that alkaline phosphatase could be extracted by means of water from both epiphyseal and diaphyseal bone. Manganese was detectable only in the ash from the epiphyseal extract. In any case there was no sign of preferential extraction of manganese as a congener or integral part of phosphatase.

Exp. (iv) showed clearly that the percentage of ash varies for the different portions of femur (Table 1).

Table 1. *Ash contents of various parts of an ox femur*

	Wt. of bone (g.)	Wt. of ash (g.)	Percentage ash
Epiphyseal portions (near head)	118	37	31
	97	26	27
	157	51	32
	153	52	34
Diaphyseal (shaft)	23	13	57
	23	13	57
Mixed type	95	37	39
	142	54	38

Exp. (v) (*a*) yielded 14·4 g. of ash from the percolate and 2·8 g. of ash from the residue in the tube (i.e. 57·2 % ash on the 30 g. used). The manganese content of the percolate was estimated, by a colorimetric procedure using periodate oxidation, to be 8 μg. The bone shavings were estimated to contain 15 μg. so that approximately half the manganese was retained. Similarly, in Exp. (v) (*b*), 16·6 g. of shavings yielded 9·3 g. of ash from the percolate and 0·31 g. from the residue, and it is estimated that approximately one-fifth of the total manganese was retained in the latter. In Exp. (v) (*c*) 56 % of ash was obtained altogether, and within experimental error the manganese was extracted with the calcium phosphate, at first slowly and then more quickly. The proportion of the total manganese remaining in the translucent residue was quite small, but expressed in p.p.m. on the ash it was high (100 p.p.m.).

The diaphyseal bone ash contained 1·44 p.p.m. (± 5 %) of manganese and the epiphyseal bone ash 1·41 p.p.m. (± 15 %). Now the fresh diaphyseal bone contains 57 % of ash so that the manganese content is about 0·51 p.p.m. The fresh epiphyseal bone contains 31 % of ash corresponding with about 0·27 p.p.m. of manganese. Thus, although the manganese content of bone varies along its length, this is due entirely to the varying amount of calcium phosphate deposited. It may be concluded that most of the manganese in ox bone is deposited with the inorganic salts, but a small proportion is definitely associated with the organic matrix.

Compared with the total, the amount of manganese (if any) which is attached to water-soluble phosphatases is negligibly small.

SUMMARY

1. Spectrographic studies and determinations based on periodate oxidations show that the manganese content of ox femur is of the order 0·27–0·51 p.p.m. depending on the type of bone, compact or cancellous. Irrespective of the type this corresponds to about 1·4 p.p.m. expressed on the ash.

2. Most of the manganese is associated with the inorganic matrix, but a small proportion is bound to the organic matrix.

3. Little manganese, if any, accompanies the water-soluble alkaline phosphatase.

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Manganese in Rabbit Tissues

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Although many papers have appeared on the manganese contents of the tissues of various animals, in no case has a survey been recorded of the concentrations in all the tissues of a single animal.

Such a survey might provide a better clue to function than comparisons of the manganese contents of tissues from different individuals or species.

EXPERIMENTAL

Animals and materials

The rabbit was chosen for this investigation because it is of a convenient size for obtaining reasonable amounts of small bodies such as the pituitary and pineal glands. Four animals were used; samples of most tissues were obtained from an adult female chinchilla and of the other tissues from the remaining three. Specimens of incompletely separated adrenal cortex and medulla, thymus and thyroid were obtained from a resting adult female chinchilla; specimens of mammary gland, thyroid and lung were obtained from a lactating female albino, and samples of pancreas, thyroid, testes and spinal cord from an adult male chinchilla. Liver samples from each animal were used as controls. The pineal and pituitary glands from the last two animals were examined. The separation of the duodenal mucosa and muscularis tissue was only approximate. All dissections were made with carefully cleaned stainless-steel instruments.

Using portions of kidney and liver from the first rabbit, homogenates were prepared and dialysed in cellophan bags against changing glass-distilled water for 10 days, toluene being added as preservative. The residue in each bag was divided into two portions and the Mn content of each determined.

Method of analysis

Mn was determined by a modification (Fore & Morton, 1952*a*) of a method described by Kun (1947) based on the catalytic oxidation of diethylaniline by Mn^{++} and periodate to give a coloured product absorbing at 470 mμ. The experimental error is from ± 10 to ± 20% depending on the size of the sample.

RESULTS

The findings on rabbit tissues are recorded in Table 1. The manganese contents based on fresh tissue weight are listed in decreasing order in Table 2. For comparison, (*a*) the values reported in the literature for each rabbit tissue have been averaged, and (*b*) the values reported for each type of tissue irrespective of species have been averaged. The published figures for the rabbit were obtained from papers by Bertrand & Medigreceanu (1912*a*, *b*; 1913), Ellis, Smith & Gates (1947), Lorenzen & Smith (1947), Lund, Drinker & Shaw (1921), Ray (1938) and Richards (1930). The figures in the right-hand column of Table 2 are averages obtained by attaching the same weight to all the published figures which can be traced. The number of papers consulted is about thirty-five and a full list of references would take up too much space.

The results of the dialyses are given in Table 3, and if the figures 2·0 and 1·2 p.p.m. are accepted for the manganese contents of the liver and kidney homogenates, respectively, it appears that in the liver 74% of the manganese, 72% of the dry matter

Table 1. *Experimental data on rabbit tissues*

Tissue	Fresh tissue wt. (g.)	Dry matter (%)	Ash as % of fresh tissue	Ash as % of dry tissue	Manganese content in p.p.m. in Fresh tissue	Manganese content in p.p.m. in Dried tissue	Manganese content in p.p.m. in Ash
Pituitary	0·0078	12	—	—	3·9 | 2·4	34 | 17	—
Pituitary	0·0071	14	—	—	1·4 | 2·4	9·7 | 17	—
Pituitary	0·0063	24	—	—	1·8 | 2·4	7·6 | 17	—
Grey hair	0·713	90	1·5	1·6	0·99	1·1	67
White hair	0·816	87	0·56	0·65	0·98	1·1	170
Hide	1·32	46	0·72	1·6	0·38	0·82	53
Fascia	4·97	23	1·1	4·7	0·024	0·10	2·2
Spleen	1·51	22	1·0	4·5	0·22	0·97	21
Bone marrow	0·640	53	0·99	1·8	0·045	0·087	4·7
Ileum	0·723	18	1·1	6·0	1·7	9·6	160
Kidney (one)	7·11	26	1·3	4·9	1·2	4·7	97
Duodenum	1·41	20	1·2	6·1	1·1	5·6	92
Mucosa	1·03	18	1·2	6·8	1·6	8·6	130
Muscularis	2·22	17	0·91	5·5	0·50	3·1	55
Salivary gland	1·42	22	1·6	7·1	1·4	6·2	88
Stomach	1·75	23	1·1	4·7	1·0	4·4	95
Caecum	1·08	17	0·79	4·7	0·82	4·9	100
Bile	1·61	20	1·7	8·5	0·48	2·4	28
Heart	1·06	22	1·0	4·5	0·28	1·3	28
Liver	3·84	30	1·6	5·3	2·0 | 2·1	6·7 | 7·9	130 | 160
Liver	3·03	29	1·5	5·3	2·2 | 2·1	7·6 | 7·9	140 | 160
Liver	2·79	24	1·2	4·9	2·0 | 2·1	8·4 | 7·9	170 | 160
Liver	3·29	25	1·2	4·9	2·2	8·7	180
Pancreas	0·225	74	1·1	1·5	1·6 | 1·6	2·1 | 4·2	140 | 130
Pancreas	0·297	26	1·5	5·8	1·6 | 1·6	6·2 | 4·2	110 | 130
Adrenals (two)	0·342	49	1·5	3·1	0·67	1·3	44
Cortex	0·290	42	1·1	2·7	0·56	1·3	50
Medulla	0·027	39	—	—	0·60	1·5	—
Ovaries (two)	0·684	23	1·5	6·3	0·60	2·6	41
Mammary gland	1·88	62	0·31	0·50	0·25	0·41	82
Mammary gland (lactating)	0·738	45	1·2	2·7	2·2	4·8	180
Brain	2·75	22	1·5	6·9	0·36	1·6	24
Muscle	2·07	23	1·2	5·0	0·13	0·56	11
Pineal	0·0061	15	—	—	3·8 | 2·3	26 | 15	—
Pineal	0·0018	—	—	—	0·83 | 2·3	3·3 | 15	—
Gall bladder	0·431	27	1·7	6·6	0·91	3·4	53
Nerve	0·178	41	1·4	3·5	0·086	0·21	6·1
Thyroid	0·0798	34	1·3	3·7	0·29 | 0·24	0·84 | 0·76	23 | 42
Thyroid	0·122	33	0·25	0·75	0·21 | 0·24	0·64 | 0·76	85 | 42
Thyroid	0·112	26	1·3	4·7	0·21 | 0·24	0·79 | 0·76	17 | 42
Testes (two)	0·0421	32	—	—	0·36	1·2	—
Spinal cord	0·393	31	1·7	5·5	0·39	1·3	23
Lung	0·868	18	0·99	5·4	0·011	0·061	1·1
Thymus	0·018	67	—	—	0·45	0·67	—

and 92 % of the ash was dialysable. In the kidney 41 % of the manganese, 49 % of the dry matter and 88 % of the ash was dialysable.

DISCUSSION

Although simple distribution studies of elements or compounds have in some instances provided valuable clues to specialized function the figures given in Table 2 do not indicate a localized role. The ubiquitous occurrence of manganese in the animal body suggests a role in general cell metabolism as opposed to metabolic processes characteristic of particular tissues. Such a role for manganese would not of course exclude other roles restricted to those tissues with a specially higher manganese content. Dialysis of kidney and liver homogenates showed that some of the manganese is loosely held and some of it very firmly combined. If it is assumed that in all tissues having somewhat higher manganese contents, a portion of the manganese is dialysable, then the already narrow range for total manganese (Table 2) becomes even narrower for the 'bound' manganese. The small range of manganese concentrations is consistent with a general metabolic role. The fact that little or no dialysable manganese is present in ox retinas which contain only 0·13 p.p.m. of manganese (see Fore & Morton, 1952*c*) supports the idea that it is only those tissues relatively rich in manganese which contain the element in dialysable form.

Indications have been obtained in other work (Fore & Morton, 1952*b*) that some of the manganese in bone and bone marrow is attached to the cell membranes. It is therefore interesting to consider the average proportion of manganese to protein in the tissues. If 0·5 p.p.m. (Table 2) is taken as the approximate tissue content of non-dialysable protein would need to have a molecular weight of the order 10^6. This figure, though large, is not impossibly so since, for example, the muscle protein myosin has a molecular weight of that order. The concept of manganese attached to the cell membrane is not entirely new. Albert, Rubbo, Goldacre & Balfour (1947) suggested that oxine inhibited bacterial

Table 2. *Rabbit tissues arranged in order of decreasing manganese content*

Tissue	Mn content in fresh rabbit tissue (p.p.m.)	Averages of figures recorded in the literature for rabbit	Average of figures recorded in the literature for a wide range of species	Tissue	Mn content in fresh rabbit tissue (p.p.m.)	Averages of figures recorded in the literature for rabbit	Average of figures recorded in the literature for a wide range of species
Bone (long)	—	3·5	3·3	Adrenal medulla	0·60	—	—
Pituitary	2·4	—	2·5	Adrenal cortex	0·56	—	—
Pineal	2·3	—	—	Duodenal muscularis	0·50	—	—
Mammary gland (lactating)	2·2	—	0·26	Bile	0·48	—	0·90
				Thymus	0·45	—	1·6
Liver	2·1	1·9	2·5	Spinal cord	0·39	—	0·60
Ileum	1·7	—	—	Hide	0·38	0·20	0·40
Duodenal mucosa	1·6	—	—	Intestine	—	0·38	0·45
Pancreas	1·6	2·3	1·9	Testes	0·36	0·40	0·50
Salivary gland (submaxilliary)	1·4	—	1·1	Brain	0·36	0·60	0·40
				Uterus	—	0·35	0·33
Kidney	1·2	1·1	1·2	Heart	0·28	0·21	0·34
Duodenum	1·1	—	1·9	Mammary gland (resting)	0·25	—	0·14
Stomach	1·0	0·90	0·80				
Colon	—	1·0	0·75	Thyroid	0·24	—	0·55
Hair	0·99	1·2	2·4	Spleen	0·22	0·55	0·40
Gall bladder	0·91	1·0	1·1	Muscle	0·13	0·14	0·18
Caecum	0·82	0·43	0·43	Milk	—	—	0·045
Salivary gland (parotid)	—	—	0·70	Bone marrow	0·045	—	<0·050
				Blood	—	0·03	0·040
Intestine (small)	—	0·70	0·45	Fascia	0·024	—	—
Adrenals	0·67	—	0·40	Lung	0·011	0·47	0·20
Lymph nodes	—	—	0·65	Urine	—	0·00	0·004
Ovaries	0·60	—	0·55	Parathyroid	—	—	—

Table 3. *Retention of manganese after dialysis of rabbit liver and kidney homogenates*

Wt. of tissue homogenate (g.)	Dialysis residue		Ash on dry matter (%)	Mn from sample (μmg.)	Mn (p.p.m.) on	
	Dry wt. (g.)	Ash wt. (mg.)			Dry wt.	Ash wt.
13·9 g. liver	0·593	8·6	1·5	4060	6·9	470
	0·583	9·6	1·6	3410	5·8	360
Totals	1·177	18·2	—	7470	—	—
Means	—	—	**1·6**	—	**6·4**	**420**
5·67 g. kidney	0·342	4·5	1·3	2270	6·6	510
	0·402	4·8	1·2	1750	4·4	360
Totals	0·744	9·3	—	4020	—	—
Means	—	—	**1·3**	—	**5·5**	**440**

manganese and 10% as an average for protein in tissue, then the proportion of manganese with respect to the protein is 5 p.p.m. There will thus be 55 parts of manganese to 11 million parts of protein and assuming one (or two) molecules of protein per atom of manganese a protein of very high molecular weight is implied. If the manganese is, however, preferentially attached to the cell-wall protein such growth by combining with essential metals in the bacterial surface, and Gale (1949) has shown that oxine inhibits glutamic acid assimilation in bacteria by combination with a metal in the cell, probably manganese.

The relatively high manganese contents of liver and lactating mammary gland could be accounted for, at least in part, by a high arginase content, and

the somewhat high manganese content in the tissues of the intestinal tract might similarly be attributed to manganese present as part of the peptidase system.

SUMMARY

1. The manganese contents of rabbit tissues have been determined, and the results compared with those obtained by previous workers.

2. The range covered by the results is narrow, especially as some of the tissues relatively rich in manganese contain much of it in dialysable form.

3. It is suggested that manganese participates in general cell metabolism while attached to the cell-wall protein.

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Manganese in Eye Tissues

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As part of a programme of research on the biochemistry of vision the manganese contents of the eye tissues of the ox have been determined. The only similar study appears to be that of Tauber & Krause (1943), who used an extension of the periodate method (Wiese & Johnson, 1938) on samples obtained by taking tissues from the eyes of a large number of oxen. The method used here is the modified catalytic method (Fore & Morton, 1952*a*); it is sufficiently sensitive to be applicable to the tissues from single eyes.

EXPERIMENTAL

Left and right eyes from each of two oxen were studied separately. After removing muscle, etc., attached to the sclera, each eye was dissected using carefully cleaned stainless-steel instruments. Separation of the tissues was complete except perhaps for the conjunctiva, and minute fragments of iris tissue were sometimes present in the sample of vitreous humour. The optic nerve was cut out of the sclera, leaving no nerve tissue in the sclera sample, from which choroid pigment was carefully removed. The cornea, lens, sclera, optic nerve and conjunctiva were washed with glass-distilled water and touched on filter paper (Whatman no. 50) before weighing. The retina, choroid and the vitreous and aqueous humours were placed directly into crucibles. The iris, which adheres to the delicate membrane retaining the vitreous humour, was touched on filter paper to remove traces of vitreous humour.

In a later experiment a retinal homogenate was prepared from about forty ox retinas, toluene added and the brei dialysed against changing glass-distilled water. The Mn contents of samples removed from the cellophan bag at 70 and 180 hr. were determined by the modified catalytic method.

RESULTS

The results of the manganese determinations of the eye tissues are given in Tables 1 and 2. In Tables 3–5 the tissues are arranged in order of decreasing manganese content alongside the values obtained by Tauber & Krause, who did not examine the aqueous humour nor the contents on an ash-weight basis.

The results of the dialysis experiment are given in Table 6. The weight of the homogenate used for dialysis expressed as dry weight (*) was calculated using the percentage dry weight (not given) obtained for two samples of the homogenate. Then, from the manganese content of these samples and the calculated dry weight, the absolute amount of manganese in the homogenate used for the dialysis (†) was obtained.

Table 1. *Experimental data on ox eye tissues*

Tissue	First experiment: Wt. of fresh tissue (g.)	First experiment: Dry matter (%)	First experiment: Ash on fresh tissue (%)	First experiment: Mn from tissue (μmg.)	Second experiment: Wt. of fresh tissue (g.)	Second experiment: Dry matter (%)	Second experiment: Ash on fresh tissue (%)	Second experiment: Mn from tissue (μmg.)
Conjunctiva	1·10*	20	0·39	286	0·169	19	1·0	21·0
	0·601*	30	0·55	164	0·085	19	0·83	19·6
Cornea	0·723	18	0·57	33·4	0·666	22	0·95	26·3
	0·788	18	0·72	Lost	0·644	23	0·89	27·8
Aqueous humour	1·25	1·3	0·85	12·8	1·62	1·2	0·94	21·8
	0·937	1·3	0·86	14·3	1·63	1·2	0·88	15·7
Iris	0·678	16	0·77	71·0	0·860	18	0·92	113
	0·821	14	0·93	104	0·838	18	1·2	91·0
Lens	2·54	34	0·68	31·6	2·41	35	0·70	16·7
	2·39	40	0·73	29·1	2·36	36	0·71	24·8
Vitreous humour	13·8	1·2	0·86	110	16·8	1·1	0·81	83·5
	12·2	1·3	0·87	163	16·2	1·1	0·82	104
Retina	0·624	11	0·87	112	0·760	12	0·90	76·8
	0·656	11	0·93	91·5	0·715	13	1·0	71·7
Choroid	0·514	18	1·07	69·1	0·480	21	1·3	49·8
	0·429	18	1·21	57·8	0·445	20	1·4	45·3
Sclera	5·15	27	0·48	675	5·58	30	0·43	1010
	5·00	29	0·54	1130	5·42	30	0·53	920
Optic nerve	0·463	28	1·06	36·8	0·639	28	1·2	9·0
	0·399	32	1·15	39·8	0·962	27	1·2	12·9

* Possibly contaminated with sclera.

Table 2. *Manganese and ash contents of ox eye tissues determined in the present work and compared with the findings of Tauber & Krause* (1943)

(Figures in brackets are the extreme values for individual determinations.)

Tissue	Ash on fresh tissue (mean) (%)	Manganese (p.p.m.) of: Fresh tissue: Present work	Fresh tissue: Tauber & Krause	Dried tissue: Present work	Dried tissue: Tauber & Krause	Ash
Conjunctiva	0·70	0·23 (0·13–0·27)	0·10 (0·069–0·11)	1·0 (0·68–1·3)	0·28 (0·23–0·37)	39 (12–67)
Cornea	0·80	0·044 (0·04–0·046)	0·11 (0·094–0·13)	0·24 (0·18–0·29)	0·44 (0·37–0·49)	6·4 (4·2–8·2)
Aqueous humour	0·89	0·012 (0·010–0·015)	—	0·98 (0·78–1·2)	—	1·5 (1·1–1·8)
Iris	0·93	0·12 (0·11–0·13)	0·24 (0·21–0·29)	0·73 (0·62–0·89)	0·92 (0·79–1·2)	13 (9–14)
Lens	0·71	0·010 (0·007–0·012)	0·18 (0·14–0·22)	0·031 (0·02–0·037)	0·52 (0·41–0·63)	1·5 (1–1·8)
Vitreous humour	0·85	0·008 (0·005–0·013)	0·031 (0·019–0·042)	0·69 (0·44–1·1)	1·5 (0·93–2·1)	0·95 (0·6–1·5)
Retina	0·93	0·13 (0·10–0·18)	0·32 (0·24–0·39)	1·1 (0·8–1·6)	2·6 (2·0–3·2)	15 (10–21)
Choroid	1·2	0·12 (0·10–0·14)	0·21 (0·17–0·32)	0·63 (0·50–0·76)	0·71 (0·59–1·1)	9·9 (5·6–13)
Sclera	0·50	0·18 (0·13–0·23)	0·09 (0·078–0·13)	0·61 (0·48–0·77)	0·32 (0·25–0·42)	36 (27–43)
Optic nerve	1·1	0·052 (0·013–0·10)	0·26 (0·22–0·31)	0·18 (0·049–0·31)	1·0 (0·83–1·2)	4·7 (1·1–8·7)

Table 3. *Order of decreasing manganese content (ox eyes) based on fresh tissue*

First ox		Second ox		Tauber & Krause	
Tissue	p.p.m.	Tissue	p.p.m.	Tissue	p.p.m.
Conjunctiva	0·27	Conjunctiva	0·18	Retina	0·32
Sclera	0·18	Sclera	0·18	Optic nerve	0·26
Retina	0·16	Iris	0·12	Iris	0·24
Choroid	0·14	Retina	0·10	Choroid	0·21
Iris	0·12	Choroid	0·10	Lens	0·18
Optic nerve	0·090	Cornea	0·041	Cornea	0·11
Cornea	0·046	Optic nerve	0·014	Conjunctiva	0·099
Aqueous humour	0·012	Aqueous humour	0·012	Sclera	0·090
Lens	0·012	Lens	0·009	Aqueous humour	—
Vitreous humour	0·011	Vitreous humour	0·006	Vitreous humour	0·031

Table 4. *Order of decreasing manganese content based on dried tissue*

First ox		Second ox		Tauber & Krause	
Tissue	p.p.m.	Tissue	p.p.m.	Tissue	p.p.m.
Retina	1·4	Aqueous humour	0·95	Retina	2·6
Conjunctiva	1·1	Conjunctiva	0·94	Aqueous humour	—
Aqueous humour	1·0	Retina	0·82	Vitreous humour	1·5
Vitreous humour	0·87	Iris	0·67	Optic nerve	1·0
Iris	0·78	Sclera	0·59	Iris	0·92
Choroid	0·75	Vitreous humour	0·50	Choroid	0·71
Sclera	0·62	Choroid	0·50	Lens	0·52
Optic nerve	0·30	Cornea	0·19	Cornea	0·44
Cornea	0·29	Optic nerve	0·050	Sclera	0·32
Lens	0·036	Lens	0·025	Conjunctiva	0·28

Table 5. *Order of decreasing manganese content (ox eye tissues) based on ash*

First ox		Second ox	
Tissue	p.p.m.	Tissue	p.p.m.
Conjunctiva	58	Sclera	37
Sclera	34	Conjunctiva	20
Retina	18	Iris	12
Iris	14	Retina	11
Choroid	12	Choroid	6·7
Cornea	8·2	Cornea	4·5
Optic nerve	8·1	Aqueous humour	1·4
Lens	1·8	Optic nerve	1·2
Aqueous humour	1·5	Lens	1·1
Vitreous humour	1·2	Vitreous humour	0·70

Table 6. *Dialysis of a retina homogenate prepared from ox eyes*

Sample	Sample (%)	Dry wt. (g.)	Ash wt. (mg.)	Mn (μmg.)	Mn content (p.p.m.) in Dry tissue		Mn content (p.p.m.) in Ash	
Homogenate portions used for control	—	0·186 0·174	12·8 12·2	248 183	1·33 1·05	1·19	19·4 15·0	17·2
To dialysis	—	2·23*	—	2650†	—		—	
70 hr. samples	27 28	0·534 0·521	10·0 8·1	783 909	1·47 1·74	1·61	78·3 112	95·2
180 hr. samples	23 22	0·393 0·368	6·8 4·8	799 689	2·03 1·88	1·96	117 143	130
Totals	100	—	—	3180	—		—	

* Estimated dry weight of homogenate used for dialysis.
† Mn content of homogenate used for dialysis calculated from analysis of portion used for control.

DISCUSSION

As both eyes in an animal receive the same blood supply a difference between the manganese contents of a given tissue from the two eyes is not to be expected. Such differences were, however, observed, and in one or two cases were probably real and not due to experimental error. For the majority of the tissues the mean values for each of the two animals showed greater differences. In no case where a different value is given (Table 3) for the two oxen did the values for the two eyes from each overlap.

This apparent difference between individual oxen cannot be used to explain the poor agreement between the values obtained in this laboratory and those of Tauber & Krause. With the exceptions of the sclera and conjunctiva the values obtained by the American workers are several times larger. The values for the conjunctiva given in the present paper are to be considered with caution as there is some doubt as to whether conjunctiva alone was included in the samples.

In regard to which of the fresh tissues contain the greatest concentrations of manganese there is fair agreement with the American results if the sclera and conjunctiva be omitted.

The retina appeared to be one of the richest tissues with respect to manganese and, as this tissue was also the site of the chemical visual processes, fractionation was attempted. When dialysis was applied to retinal homogenate more manganese was recovered in the non-dialysable material than was present (by calculation) in the original homogenate. No explanation of this can be offered since rigorous precautions against contamination were taken. However, it appears from the experiment that little of the manganese in the retina is dialysable.

The manganese in retina appeared to be attached to the retinal tissue and the latter was therefore fractionated, using a well defined technique in use in this laboratory, to give preparations containing most of the rods, the photoreceptors of scotopic vision. There appeared to be a smaller concentration of manganese in the rods than in the remainder of the retina. Hence there is so far no evidence that the manganese present in the retina is selectively or specificially concerned with visual biochemistry. The functions of manganese present in the eye tissues may well be displayed at other sites in the body (see Fore & Morton, 1952*b*, on rabbit tissues).

SUMMARY

1. The manganese contents of the tissues of single ox eyes have been determined.

2. The results have been compared with those of Tauber & Krause (1943) obtained using large samples from several hundred ox eyes.

3. Preliminary experiments on the fractionation of retinas have indicated that manganese in this tissue is non-dialysable and not specifically concerned in the visual process.

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The Determination and Distribution of Phosphocreatine in Animal Tissues

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Phosphocreatine has been described for over 20 years and methods for its determination in tissues generally depend upon the estimation of the phosphorus moiety which can be split off readily in acid solution. A much-used technique is that described by LePage & Umbreit (1945), whereby the inorganic P is determined by the method of Fiske & Subbarow (1925) after its precipitation from an alkaline solution as a barium or calcium salt. The difference between this value and that determined by the same method prior to precipitation, is assumed to give the phosphocreatine P. This method, which lacks the sensitivity desirable if low phosphocreatine concentrations are present, has the further disadvantage that its accuracy depends on the quantitative precipitation of inorganic P under the recom-

mended conditions (cf. Ennor & Stocken, 1948*a*) and upon the assumption that phosphocreatine is the only organophosphate hydrolysed by acid molybdate. There is, however, no evidence for this assumption, and the work of Weil-Malherbe & Green (1951) shows that other organophosphates may, in fact, be hydrolysed.

Another technique for the determination of phosphocreatine depends on the estimation by means of the Jaffe reaction of the creatinine resulting from a 20 min. autoclaving of the sample in acid solution (McIlwain, Buchel & Cheshire, 1951).

A much more direct and rapid method, possessing a high degree of sensitivity, is to determine the creatine moiety as such. For this purpose the method described by Ennor & Stocken (1948*b*) is suitable, since it allows the determination of creatine in an alkaline medium in which phosphocreatine is perfectly stable. The creatine bound as phosphocreatine can then be determined after acid hydrolysis. The use of this method for the determination of phosphocreatine in tissue extracts and some analytical results on the distribution of phosphocreatine in various tissues will be described. In addition, a comparison has been made of the values obtained by this method, and the commonly used technique of determining the acid-molybdate-labile P.

EXPERIMENTAL

Removal of tissues. The animals used were well fed, and in order to reduce possible losses of phosphocreatine due to anoxia, all tissues were removed under surgical anaesthesia induced by the intraperitoneal injection of nembutal. The kidney, spleen and testis were exposed, with minimal handling, and a loose ligature placed around the vascular supply. The ligature was then tightened and simultaneously the vessels between the ligature and the organ were severed. The organ was then dropped into liquid O_2. Loose ligatures were placed around both ends of the gastrocnemius, and as these were tightened the muscle was simultaneously excised and dropped into liquid O_2. The abdominal incision was then temporarily closed, an incision made in the neck and the trachea exposed and opened. A tracheal cannula was inserted and connected to a respiration pump. Samples of brain were then obtained, either by the technique described by Kerr (1935), or by the rapid excision of a portion of the unfrozen brain which was then dropped into liquid O_2. The abdomen was then quickly re-opened and the incision extended into the thoracic cavity in such a way as to expose the heart. A lobe of liver was then excised, the heart removed and the two samples dropped into liquid O_2. The maximum time which elapsed between the removal of an organ or tissue from its natural environment and its immersion in liquid O_2 was 2 sec.

Preparation of tissue extracts. Trichloroacetic acid extracts of all tissues, with the exception of skeletal muscle, were prepared as described by Ennor & Stocken (1948*a*). In the case of skeletal muscle, frozen samples were dropped into the bowl of a tared Nalco type homogenizer, which was then re-weighed. Two volumes of 10% (w/v) trichloroacetic acid were then added and the tissue homogenized. The suspension was then centrifuged and the residue, after removal of the supernatant, re-extracted with an additional volume of 5% (w/v) trichloroacetic acid. The supernatants were then combined, adjusted to pH 7·0–7·3 by the addition of 5 N-NaOH and made to known volume. During these and subsequent operations the temperature of the extracts was kept at 0°.

Determination of 'free' and 'bound' creatine. In this paper, 'free' creatine refers to creatine determined directly on a sample of a neutralized extract by the method described by Ennor & Stocken (1948*b*), using the reagents and concentrations described by Eggleton, Elsden & Gough (1943). Since phosphocreatine has no free amino group it is non-reactive and is, moreover, perfectly stable under the alkaline conditions under which colour development occurs. 'Total' creatine is the sum of 'free' creatine and that which is released following acid hydrolysis. The difference between 'total' creatine and 'free' creatine gives the 'bound' creatine, which is assumed to have its origin in phosphocreatine.

For the determination of total creatine, a sample was treated under mild conditions (9 min. in 0·1 N-HCl at 65°) to hydrolyse the phosphocreatine with minimal formation of creatinine (cf. Barker, Ennor & Harcourt, 1950). The solution was then neutralized by the addition of the theoretical

Table 1. *Phosphocreatine distribution in normal animals*

(All values are expressed as mg./100 g. wet weight and are the means of duplicate determinations.)

Animal	Spleen	Kidney	Liver	Cardiac muscle	Skeletal muscle	Brain	Testis
Rabbit 1	0·8	1·6	3·2	30·2	142	—	—
2	0·8	0	6·1	3·2	97	—	—
3	5·1	0	0	0	323	0	—
4	4·2	2·1	0	29·0	—	4·8	—
Cat 1	8·5	6·1	17·0	54	222	—	—
2	7·2	19·4	24·5	82	342	—	—
3	5·2	3·7	14·8	9·7	156	41·8	10·5
4	3·2	—	0	14·4	194	22·6	—
Guinea pig 1	4·0	1·6	0·3	60	157	—	14·9
2	4·4	5·3	1·8	—	—	0	5·8
3	—	0·4	1·0	—	—	34·1	—
4	—	2·3	—	0	100	42·7	—
Dog	5·2	4·4	1·8	45	251	—	5·6

amount of NaOH and cooled rapidly to room temperature by immersion in an ice bath. In practice, a 1·0 ml. sample was pipetted into a tube graduated at 10 ml., 2·0 ml. of water were added and the contents equilibrated at 65°. 1·0 ml. of 0·4 N-HCl was then added and, after 9 min., 1·0 ml. of 0·4 N-NaOH. After cooling, 1·0 ml. of a 0·05 M solution of sodium *p*-chloromercuribenzoate was added, followed by the diacetyl and 1-naphthol. The volume was adjusted to 10·0 ml. by the addition of water and the contents mixed and allowed to stand in the dark for 15 min. for colour development. The colour intensities were measured at the same time interval as the standards which contained 10–40 μg. creatine. All determinations of free and total creatine were carried out on the day of preparation of the extract.

Using this technique, which has been shown by Barker & Ennor (1951) to result in excellent recoveries of both added creatine and phosphocreatine, analyses have been carried out on the phosphocreatine contents of various tissues from a number of different species of laboratory animals.

RESULTS

The results show that phosphocreatine was present in all tissues examined (Table 1), although there was a considerable variation in the absolute amount. Skeletal muscle, as has been found by other workers, contained much greater amounts than any other tissue. The variation in phosphocreatine content of cardiac muscle was much greater than would have been expected on the basis of analogy with skeletal muscle. No explanation is available for the occasional very low values for phosphocreatine, but it is doubtful if they are due to any rapid degradation of the compound, since the heart was, in all cases, beating vigorously when dropped into liquid O_2.

It is difficult to compare these results with those of other workers because, almost without exception, it has been the custom to determine molybdate-labile P rather than the creatine moiety of phosphocreatine (cf., however, McIlwain *et al.* 1951). Further comment on such procedures is made below, but because of the variation in phosphocreatine content in different organs it was considered of interest to compare the free and total creatine contents and to determine the percentage of the total creatine which is bound as phosphocreatine. This has been done in Table 2.

The concentration of total creatine found in skeletal muscle is much greater than in any other tissue. On the other hand, the proportion which is phosphorylated, whilst greater and showing much less variation in muscle than in any other tissue, is not as great as would be anticipated on the basis of the difference in phosphocreatine levels. If one neglects the values less than 10 % the figures suggest that, irrespective of the tissue, the proportion of creatine phosphorylated is about 20 % of the total amount present.

Table 2. *Distribution of free and total creatine in normal animals*

(All figures are the means of duplicate determinations. F = mg. free creatine/100 g. wet wt.; T = mg. total creatine/100 g. wet wt.; % B = % of creatine bound as phosphocreatine.)

Animal	Spleen			Kidney			Liver			Cardiac muscle			Skeletal muscle			Brain			Testis		
	F	T	B (%)	F	T	B (%)	F	T	B (%)	F	T	B (%)	F	T	B (%)	F	T	B (%)	F	T	B (%)
Rabbit 1	4·7	5·2	9·6	4·1	5·1	2·0	4·2	6·2	32	93	112	17·0	322	410	21·5	—	—	—	—	—	—
2	5·9	6·4	7·8	4·6	4·6	0	10·4	14·2	27	103	105	1·9	322	382	15·7	—	—	—	—	—	—
3	9·0	12·2	26	29·3	29·3	0	21·4	21·4	0	131	131	0	239	440	23	101	101	0	—	—	—
4	7·6	10·2	26	11·3	12·6	10·3	15·5	15·5	0	114	132	13·6	—	—	—	101	104	2·9	—	—	—
Cat 1	8·9	14·2	37·2	11·8	15·6	24·4	41·1	51·6	20·4	169	203	16·8	324	462	30	—	—	—	—	—	—
2	8·4	12·9	35	28·2	40·3	30	30·9	55·2	27·8	149	200	25·5	219	432	26	—	—	—	—	—	—
3	7·2	10·4	30	12·1	14·4	16	35·6	44·8	20·5	105	110·7	5·2	97	193·6	50	104	129·6	19·8	69	75·5	9·4
4	5·9	9·0	35	—	—	—	33·5	33·5	0	182	191·3	4·9	195	315	38	115	128·7	10·6	—	—	—
Guinea pig 1	—	—	—	14·2	15·6	9·0	—	—	—	48·7	48·7	0	216	278	22	71·0	97·5	27	—	—	—
2	7·4	10·1	26	16·5	19·8	16·6	5·5	6·6	16·7	—	—	—	—	—	—	121	121	0	32	35·6	10·1
3	—	—	—	8·1	8·4	2·4	8·3	9·3	10·7	—	—	—	—	—	—	60·0	80	25	—	—	—
4	10·4	12·9	19·4	7·6	8·6	23·3	4·4	4·6	4·3	80·7	118	31·5	341	439	22·3	—	—	—	27·7	37·0	25·0
Dog	9·4	12·6	25·4	11·6	14·3	18·9	2·8	3·9	28	240	268	10·3	214	370	42	—	—	—	174	183	5·5

Since phosphocreatine is usually determined by measurement of the P split off in acid molybdate, it is of interest to compare the results so obtained with those obtained by the present method. For this purpose, inorganic P was determined on one sample by the method described by Ennor & Stocken (1950). Another sample was then allowed to stand in acid molybdate at 30° for 30 min.; under these conditions, authentic phosphocreatine is completely hydrolysed. At the end of the hydrolysis period the resultant phosphomolybdate was extracted by the usual technique of Ennor & Stocken (1950). Additional samples were taken for the determination of bound creatine, as described above, and the results (Table 3) expressed in terms of P.

Table 3. *Comparison of values obtained for phosphocreatine measured as acid-molybdate-labile* P *and as bound creatine*

(All figures are expressed as mg. P/100 g. wet weight of tissue. Found = molybdate-labile P; theory = P equiv. to bound creatine, assuming this is bound as phosphocreatine.)

	Kidney		Liver		Spleen		Brain		Testis		Skeletal muscle	
Animal	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory	Found	Theory
Guinea pig 1	4·0	0·8	0·8	0·2	—	—	0	0	—	—	—	—
2	1·6	0·1	1·7	0·4	—	—	—	—	—	—	—	—
3	1·4	0·3	0	0·3	—	—	6·3	6·3	—	—	—	—
Cat	—	—	0	0	1·4	0·4	2·1	3·3	—	—	27·5	28·5
Dog	1·6	0·7	0	0·3	2·1	0·8	—	—	2·2	0·8	40·6	36·9
Rabbit 1	1·1	0	0·7	0	1·6	0·4	1·0	0	—	—	27·6	22·5
2	1·2	0·1	1·5	0	1·4	0·3	1·8	0·4	—	—	—	—

It is clear that the molybdate-labile P found almost always exceeds that demanded by theory on the basis of the bound creatine. A closer agreement is found in the case of skeletal muscle, where the phosphocreatine content is relatively high. The method for the determination of bound creatine can be considered reliable since good recoveries of added authentic phosphocreatine were obtained by Barker & Ennor (1951). Moreover, no creatine-containing compound is known which reacts in the same way as phosphocreatine with α-naphthol and diacetyl both prior to and after mild hydrolysis. The method may then be expected to yield results which accurately represent the phosphocreatine content of a tissue. For the determination of the P moiety of phosphocreatine, using the criterion of lability in acid molybdate, no such claims can be made. Since the amount of P found generally greatly exceeds that expected on the basis of bound creatine, it must be concluded that organophosphates other than phosphocreatine are acid-molybdate-labile, and hence expression of results in terms of phosphocreatine will be erroneous. Lipmann (1941), commenting on the presence of acid-molybdate-labile phosphate in tissue, has pointed out that this does not constitute proof of the presence of phosphocreatine. Franks (1932) has made a similar observation. More recently, Weil-Malherbe & Green (1951) have shown that several organophosphates commonly occurring in tissues are acid-molybdate-labile.

DISCUSSION

The method which has been described for the determination of phosphocreatine has several advantages over existing methods. Prime amongst these is the fact that determination of free creatine is carried out in an alkaline medium in which phosphocreatine is perfectly stable. Acid hydrolysis of another sample and subsequent determination of total creatine allows calculation of bound creatine and thus of phosphocreatine. If the suggested conditions of hydrolysis are adhered to, the small amount of creatinine which is formed (Barker & Ennor, 1951) may be neglected.

The method has the additional merit that a check can be made on the accuracy by which the P of phosphocreatine is determined. This is of some importance, particularly in the case of work involving radioactive P, where contamination by P from other compounds may produce gross errors in the determination of the specific activity. However, close agreement of bound creatine with molybdate-labile P would not necessarily constitute proof of the reliability of results when radioactive P is employed. Clearly, some specific enzymic procedure is to be preferred.

It has been shown that phosphocreatine has a much wider distribution than is generally supposed, and that it was present in all tissues examined. Of especial interest is the fact that, whilst the concentration varies greatly from tissue to tissue, the proportion of the total amount of creatine present in the phosphorylated form shows much less variation. This suggests that the amount of phosphocreatine

depends on the creatine content of the tissue. The belief that the presence of phosphocreatine is almost exclusively confined to muscular and nervous organs (Eggleton & Eggleton, 1929; Gerard & Tupikova, 1939) has given rise to the concept (Lipmann, 1941) that phosphocreatine has a connexion with the speed of action required by these organs. The occurrence of phosphocreatine in such organs as spleen, liver and kidney, on which no sudden demands for energy are made comparable to that placed upon, for example, skeletal muscle, suggests the desirability for some modification of this concept.

It would seem probable that phosphocreatine has a function other than that of acting as a reservoir of readily available phosphate-bond energy for the adenylic acid system and that it may contribute energy to endergonic reactions directly and without the mediation of the latter system.

SUMMARY

1. A method is described for the determination of phosphocreatine in protein-free tissue extracts.

2. Phosphocreatine has been shown to occur in all tissues investigated and the proportion of the total creatine present which is phosphorylated is of the same order irrespective of the tissue concerned.

3. Measurement of the phosphocreatine content of tissue by determination of acid-molybdate-labile phosphorus has been shown to be subject to error.

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Histamine Metabolism in the Human Placenta and in the Umbilical Cord Blood

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In a recent paper (Kapeller-Adler, 1951) a new simple microchemical method and a new unit have been proposed for the estimation of histaminase activity in biological media. In the present work, this new method, along with the biological estimation of histamine, has been applied to the study of histamine metabolism in human placenta and in umbilical cord blood. A significant decrease in histaminase activity has been observed in placentae of cases of severe toxaemia of pregnancy and a significant increase in placentae of women with twin pregnancy. The placental histamine content of women with severe toxaemia of pregnancy has been found to be greatly increased as compared with that of normal placentae. The results are related to previous observations made upon this subject with different methods. In view of contradictory findings concerning the action of histaminase on cadaverine (Kapeller-Adler, 1951), it was thought desirable to include observations on the effect of placental histaminase on this diamine in this paper.

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METHODS

Preparations of histaminase from placenta. The placentae were obtained as soon as possible after the confinement and were worked up within 24 hr. Superficial blood was removed by rapid rinsing with cold water and drying between sheets of filter paper. After the removal of membranes and the umbilical cord the organ was weighed and finely minced in an electric mincing apparatus. Thoroughly mixed pulp (40 g.) was ground in a mortar with 200 ml. saline. The fine suspension was transferred to a conical flask, was allowed to stand for 1 hr. and then filtered through a fluted filter (Whatman no. 5). 1 ml. of the clear filtrate, corresponding to 0·2 g. placental tissue, was used for each determination. The volumetric histaminase method (Kapeller-Adler, 1951) was used.

Preparations of placental extracts for the biological estimation of histamine. 20 g. finely minced placenta are ground in a mortar with 30 ml. of 10% (w/v) trichloroacetic acid (TCA) and some silver sand, and then transferred to a conical flask. After 1 hr., the precipitate is filtered through a Büchner funnel, sucked completely dry, and then washed with 5 ml. of 10% TCA. This procedure is repeated twice, using 5 ml. of 10% TCA each time, the residue being sucked dry after each washing. The histamine content of the combined filtrate and washings is determined by the biological test of Barsoum & Gaddum (1935), as modified by Code (1937*a*). In every case, histamine was identified by tests with the antihistamine drug 'Mepyramine' (2-amino-N^2-dimethylaminoethyl-N^2-*p*-methoxybenzylpyridine) by the quantitative method described by Reuse (1948).

Simultaneous estimations of histamine in plasma and blood cells. Citrated blood (10 ml.) was centrifuged at 3000 rev./min. for 15 min. Only specimens completely free from haemolysis were tested. The plasma was very carefully separated from the cells, and the histamine content of both plasma and cells was separately determined by the biological method mentioned above. Similar histamine estimations were carried out with the blood from the umbilical cord.

The histaminase activity in umbilical cord serum. This was determined by the volumetric method (Kapeller-Adler, 1951).

RESULTS

Histaminase activity in placentae

Placentae of women who had been delivered spontaneously or by a caesarean section at or before term, after a normal or toxaemic pregnancy, were examined for histaminase activity. Table 1 shows the results obtained in these investigations.

If the average results shown in the first line of Table 1 for the histaminase activity in normal placentae, delivered at term, are expressed in terms of μg. of histamine inactivated per hr., it follows that 1 g. of placenta inactivates 45·54 μg. of histamine/hr. and the total placenta, 22·90 mg. of histamine/hr.

Table 1. *Effect of placental histaminase on histamine and cadaverine in parallel assays*

(Histaminase activity is expressed in permanganate units (P.U.) per g. placenta and per total placenta. 1 P.U. represents the amount of enzyme which, after 24 hr. at 37° and pH 7·2 in an atmosphere of O_2 with 1 mg. histamine hydrochloride as substrate and with an aqueous solution of indigodisulphonate, uses 0·1 ml. 0·002 N-$KMnO_4$. 1 P.U. corresponds to the destruction of 0·46 μg. histamine/hr. In this and succeeding tables the values given are means ± S.D. of a single observation.)

Cases		Histamine	Histamine	Cadaverine
Number	Description	(P.U./placenta)	(P.U./g. of placenta)	(P.U./g. of placenta)
63	Normal, at term	49 800 ± 10 700	99·0 ± 14·8	155·0 ± 3·2
14	35th–39th week of pregnancy	40 960 ± 12 500	90·2 ± 25·7	148·8 ± 42·1
1	20th week of pregnancy	—	122·0	145·5
13	Caesarean section 36th–40th week	36 300 ± 16 000	65·1 ± 20·6	118·6 ± 39·0
5	Mild toxaemia	38 640 ± 6 100	75·6 ± 9·6	155·8 ± 35·0
19	Severe toxaemia	22 520 ± 7 840	52·6 ± 15·9	120·3 ± 36·7
4	Twin pregnancy	98 040 ± 12 042	143·0 ± 23·6	187·5 ± 58·4
5	Twin pregnancy + toxaemia	54 920 ± 30 300	84·4 ± 60·8	143·0 ± 80·3

Placentae of women with normal pregnancy, delivered before term (35th–39th week of gestation), showed a slightly lower histaminase activity. A strikingly high histaminase content was observed in the placenta of a woman who had to be operated on in her 20th week of pregnancy. Placentae of women, delivered by a caesarean section in the 36th–40th week of pregnancy, showed a lower histaminase activity than placentae of women who had been delivered spontaneously at a similar stage of pregnancy. This observation appears to point to an increased histaminase activity at the onset of labour and during birth, and does not confirm previous findings (Danforth & Gorham, 1937; Kapeller-Adler, 1944) when a higher histaminase activity was observed in the placentae of women after a caesarean section. Somewhat reduced histaminase activity was found in the placentae of women who had suffered from mild toxaemia of pregnancy. More apparent, however, was the decrease in histaminase activity in the placentae of women with severe pregnancy toxaemia. It should be mentioned here that three out of nineteen women of this group had had stillbirths. The histaminase content of placentae of

women with normal twin pregnancy was found to be much higher than in placentae of women with normal single pregnancy. The placental histaminase activity in twin pregnancy, complicated by pre-eclamptic toxaemia, was greater than that of women with toxaemia of pregnancy and only one foetus. It was further found in this work that the histaminase activity of 1 g. of a normal placenta, delivered at term, was about ten times stronger than that of 1 ml. of maternal serum in the 40th week of gestation.

The histamine content of human placentae

It can be seen from Table 2 that, whereas no difference was found between the placental histamine values of women after a normal and after a mild toxaemic pregnancy, the histamine in

Table 2. *Histamine content of placentae*

No. of placentae	Cases	Histamine of placenta (μg./g.)
22	Normal	0·10±0·0104
4	Mild toxaemia	0·06±0·0054
11	Severe toxaemia	0·40±0·1700

placentae of cases with severe toxaemia was about four times higher than in normal placentae. The results obtained for the histamine and histaminase activity in placentae of women with severe pre-eclamptic toxaemia, as compared with the corresponding figures in placentae of women with normal pregnancy, seem to indicate that the histamine content of the placenta is inversely related to histaminase activity.

Histaminase and histamine activity in the umbilical cord blood

The serum obtained from the umbilical cord blood, tested for histaminase activity by the volumetric method, did not show any activity in any of the thirty-five examined cases. There was, however, a considerable histaminolytic effect in the maternal serum during labour (Table 3). In thirteen cases, the

Table 3. *Histaminase and histamine content of maternal and foetal blood*

Number of cases	Determination	Mother	Foetus
35	Histaminase (P.U./ml.)	7·1±0·92	0
13	Histamine (μg./l.)		
	Cells	42·3±16·1	57·7±36·0
	Plasma	34·6±12·2	32·7±19·4

histamine content of the plasma and the blood cells was simultaneously investigated in the maternal and foetal blood. Results given in Table 3 show that the histamine content of the foetal blood cells was higher than that of the maternal blood cells, and that the plasma histamine was of the same order in both the mother and the foetus. The fact that the foetal blood, unlike the maternal blood, does not show any histaminase activity, and that the foetal plasma contains a similar amount of histamine to that in the maternal plasma, must give rise to some speculation about the role of histamine during parturition. It may be worth while mentioning here that Rogers (1950) found the musculature of the human umbilical arteries to be exceedingly sensitive to histamine. It may be that histamine plays a part in the permanent occlusion of the umbilical arteries during birth. In Table 1, results are also presented which were obtained when placental histaminase acted on cadaverine and histamine in parallel assays. It follows from this table that the placental histaminase is much more active on cadaverine than on histamine. In contrast to this, histaminase made from hog kidney was found to be 4 times more active on histamine than on cadaverine (Kapeller-Adler, 1951). This table also shows that the results with cadaverine did not vary much in different types of case, while those with histamine did vary.

DISCUSSION

Danforth & Gorham (1937) were the first to report histaminase activity in the human placenta. Since then, many papers have been published on this problem (Marcou, Chiriceanu, Cosma, Gingold & Parhon, 1938; Zeller, Schär & Staehelin, 1939; Zeller, Birkhäuser, Mislin & Wenk, 1939; Effkemann & Werle, 1940; Ahlmark, 1944; Kapeller-Adler, 1944; Anrep, Barsoum & Ibrahim, 1947; Swanberg, 1948, 1950; Wicksell, 1949), but varying values for the histaminase activity in the placenta, ranging from 20 to 220 μg. of histamine inactivated per g. of placenta per hr., were obtained by different workers. The results for histaminase activity obtained in this work by a microchemical technique agree with the findings of Anrep and his co-workers (1947) who, using a biological histaminase test, observed an inactivation of 20–30 μg. of histamine per g. of placental tissue per hour. These workers also found the histaminase activity of 1 g. placenta to be 8–15 times greater than that of 1 ml. plasma. A similar observation has been made in this paper. Swanberg (1950) quotes a ratio of 20:1 for the histaminolytic power of 1 g. of placenta compared with that of 1 ml. of plasma. As regards the high histaminase concentration in the placenta of a woman delivered in the 20th week of gestation (Table 1), Swanberg (1948) and Wicksell (1949) also found that the histaminase content of the placenta rapidly increases in the first half of pregnancy. Anrep *et al.* (1947), on the other hand, concluded that placentae of different age and weight do not differ greatly in the histaminase concentration. The low activity of placental histaminase, which I have

found in cases of severe pregnancy toxaemia, does not agree with the results of Ahlmark (1944), who found more histaminase in some placentae of toxaemic women than in normal women. There are no previous reports on the incidence of high histaminase activity in the placentae of women with twin pregnancy. Since the placental histaminase in twin pregnancy was much higher, not only for the whole placenta, but also per g. of placenta as compared with results in single pregnancy, it seems as if not only the increase in the mass of placental tissue but also some qualitative differences in the tissue were responsible for the rise in histaminase activity in multiple pregnancy. It should be recalled here that Guido (1942) and Anrep *et al.* (1947) concluded that the placenta is the site of formation of histaminase during human pregnancy. According to extensive studies of Swanberg (1948, 1950), the histaminolytic enzyme is formed in the decidua.

Reports on the histamine content of the human placenta are very scanty. Wicksell (1949) investigated the histamine content of nine placentae and reported values ranging from 0·16 to 1·5 μg. histamine/g. placenta. The histamine contents of placentae reported here are much lower. The observation made in this paper that blood from the umbilical cord does not show any histaminase activity (Table 3) seems to agree well with results obtained by Swanberg (1948) and by Wicksell (1949), who both, using a biological histaminase test, found that the histaminolytic power of foetal blood was of the same order, or slightly higher, than that of non-pregnant persons. Wicksell (1949) also observed that the foetal blood contained more histamine than the maternal blood and that the foetal plasma histamine was of the same range as was the maternal plasma histamine. Wicksell explains this observation on the known fact that the amount of cellular elements is greater in the foetal than in the maternal blood and that the blood histamine mainly occurs in cells such as leucocytes and platelets (Schwartz, 1936; Code, 1937*a*, *b*; Zon, Ceder & Crigler, 1939; Minard, 1941). The results shown in Table 3 of this paper, presenting separately the histamine content of the maternal blood cells and plasma and the foetal blood cells and plasma, seem to confirm this conception.

The discrepancy in the behaviour of histaminase towards histamine and cadaverine, depending on the origin of the enzyme, observed in this work as well as in preceding papers (Kapeller-Adler, 1944, 1949, 1951) is at present under investigation.

SUMMARY

1. The histaminase activity in 124 placentae of women with normal or toxaemic pregnancy has been investigated by a microchemical method, and the results have been considered in relation to those obtained by other workers with biological methods. The histamine content of some of the placentae has also been estimated.

2. The histaminase and histamine activity in the maternal and foetal blood have been simultaneously investigated, and the role that histamine might play in the permanent occlusion of the umbilical arteries during birth has been discussed.

3. Placental histaminase has been found to be more active on cadaverine than on histamine. Kidney histaminase and histaminase from human placentae differ in the relation between their actions on histamine and cadaverine.

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The Pathway of Oxidation of Acetate in Baker's Yeast

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Lynen & Neciullah (1939), Lynen (1942, 1943), Weinhouse & Millington (1947) and Martius & Lynen (1950) have put forward the view that the tricarboxylic acid cycle represents the main pathway of oxidation of acetate in yeast cells. This hypothesis was based on the observation that the intermediary stages of the cycle can occur in yeast preparations and that the oxidation of isotopic acetate leads to the formation of isotopically labelled citrate and succinate, the isotope being in the positions expected on the basis of the hypothesis. The validity of the evidence has been questioned (Krebs, 1949, 1950) because of the very low rate at which some of the intermediates of the tricarboxylic acid cycle are metabolized.

Isotope experiments and other results reported in this paper show that acetate can be oxidized in yeast cells through a mechanism other than the tricarboxylic acid cycle, or a dicarboxylic acid cycle such as postulated by Foster *et al.* (1949). At the same time the occurrence of the component reactions of the tricarboxylic acid cycle in yeast cells is confirmed. Reasons are put forward in support of the view that in yeast cells the reactions of the tricarboxylic acid cycle serve to supply intermediates for synthetic processes but may not primarily be concerned with the production of energy.

Permeability barriers, and the inability to prepare respiring yeast extracts, present obstacles to experiments on the intermediary metabolism of yeast cells. In intact cells the rate of penetration of the di- and tri-carboxylic acids is negligible compared with the rate of respiration, but Dixon & Atkins (1913) and Lynen (1939) have shown that the permeability barriers can be overcome by exposing the cells to the temperature of liquid air or liquid nitrogen. Although cold-treatment somewhat reduces the activity of the oxidative enzymes, a considerable proportion of the oxidizing activity remains if the cold-treatment is carried out under suitable controlled conditions. The first part of this paper is concerned with the study of the properties of cold-treated cells, the second with the application of the isotope technique to the study of oxidative processes in such cells.

METHODS AND RESULTS

The great majority of the experiments was carried out on baker's yeast supplied by the Standard Yeast Co. Ltd., but some were repeated with pure cultures of Fleischmann's yeast. This was grown at 25° in Roux bottles on medium containing 10 ml. yeast extract (prepared by heating baker's yeast with an equal volume of water to 100° for 30 min. and filtering), 1 g. glucose, 2 g. casein hydrolysate ('Pronutrin') and 2 g. agar in 100 ml. The gas exchange was measured manometrically in conical Warburg manometers at 25° unless otherwise stated. In all aerobic experiments the gas space of the manometer vessels contained O_2 and the centre well 0·2 ml. 2N-NaOH to absorb CO_2. Solid CO_2, henceforth referred to as 'dry ice', was used in most experiments for cold-treatment.

Oxidation of various substrates by frozen cells. Baker's yeast which had been stored in the refrigerator was crumbled into small pieces and thoroughly mixed, and left in contract for 20 min., with an excess of dry ice. On thawing, the yeast became liquid; it was suspended in 40 parts of 0·133M-phosphate buffer, pH 6·5. Each manometer vessel contained 3 ml. yeast suspension in the main compartment and 1 ml. 0·1M-substrate solution in the side arm. The substrate was added after an equilibration period of 10 min. The O_2 uptake for the periods 40–60 min. and 80–100 min. after the addition of substrate is shown in Table 1, together with comparative data for yeast not exposed to dry ice. In the untreated cells, acetate, ethanol, glucose, D- and L-lactate and pyruvate were readily oxidized. There was considerable blank respiration which was not appreciably increased by the ten other substrates tested. Cold-treatment and washing had no effect on the rate of the blank control. Acetate, ethanol, glucose, D- and L-lactate and pyruvate all increased the O_2 uptake of treated cells, but the increase (for the 80–100 min. period) was not more than 25 % of that found in the fresh cells. Succinate which had no effect in the untreated cells markedly increased the O_2 consumption in the frozen cells. There was no definite effect of the other nine substrates. Thus, whilst the oxidation of the substrates which are generally oxidized in yeasts is slower in the cold-treated cells, succinate, though not oxidized

in untreated cells, becomes oxidizable after cold-treatment.

Dialysis for 16 hr. against running tap water had no major effect on the cold-treated yeast. The blank respiration did not appreciably decrease, nor were the rates of oxidation of acetate and succinate affected. On storage in the refrigerator the oxidative capacity fell, e.g. 50% in 48 hr.

Table 1. *Oxidation of various substrates by dry-ice-treated baker's yeast*

(For experimental conditions see text.)

	O_2 absorbed by 4 ml. suspension (40 mg. fresh wt.) (μl.)			
	Untreated cells		Dry-ice-treated cells	
Substrate added	40–60 min.	80–100 min.	40–60 min.	80–100 min.
None	61	43	69	31
Acetate	468	492	119	140
Ethanol	581	651	148	165
Glucose	638	576	172	183
D-Lactate	328	242	132	97
L-Lactate	324	211	187	123
Pyruvate	267	310	107	96
Butyrate	68	51	76	37
Formate	77	42	66	37
Glutamate	66	44	65	29
α-Ketoglutarate	62	44	77	34
L-Aspartate	62	43	75	36
Citrate	59	44	69	32
Fumarate	57	41	53	23
Tartrate	60	43	68	32
Succinate	61	45	83	55

Demonstration of increased permeability. That the non-oxidation of fumarate and citrate in cold-treated cells is not due to permeability barriers is shown by the rapid formation of L-malate and *iso*-citrate on addition of fumarate and citrate.

To test fumarase activity, dry-ice-treated yeast (10 g.) was twice washed with 100 ml. water and the sediment suspended in a volume of 100 ml. A sample (10 ml.) of this suspension, 15 ml. 0·1 M-fumarate, 0·5 ml. M-$NaHCO_3$ and 4·5 ml. water were mixed and saturated with 5% CO_2 +95% N_2 (v/v) at 25°. Samples of 5 ml. were removed at intervals for polarimetric analysis. They were mixed in quick succession with 1·25 ml. acetic acid, 6 ml. water, 2·5 ml. M-sodium citrate and 11·25 ml. 29% (w/v) ammonium molybdate. After filtration, readings were taken in 2 dm. tubes (see Krebs & Eggleston, 1943).

Time (min.)	Reading ($\alpha_D^{17°}$)	L-Malic acid (mg./ml. incubated solution)
0	0	0
15	+0·78	1·25
30	+1·34	2·34

No measurable amount of malate was formed in untreated cells. Washing removed blank rotations, but did not appreciably affect the fumarase activity. The supernatant was inactive. The fumarase activity was thus very high, compared with other metabolic processes, Q_{malate} being about 75 μl./mg. dry wt./hr. under the test conditions.

To test aconitase activity the washed, dry-ice-treated cells were suspended in an equal volume of water and 8 ml. of the suspension were mixed with 10 ml. M-sodium citrate, 6 ml. water and 1 ml. M-$NaHCO_3$ and saturated at 25° with 5% CO_2 in 95% N_2 (v/v). For the polarimetry (see Eggleston & Krebs, 1949) 8 ml. samples were used, the concentration of all the reagents being the same as in the malate determination. A little charcoal was required to obtain a clear filtrate. Readings were as follows:

Time (min.)	Readings ($\alpha_D^{17°}$)	*iso*Citric acid (mg./ml. incubated solution)
20	−0·73	1·46
40	−1·14	2·37

It follows from these data that $Q_{isocitrate}$ was about 16 μl./mg. dry wt./hr. under test conditions. Again washing with water did not reduce the enzyme activity. The absence of enzyme activity in the washing fluid shows that fumarate and citrate reacted within the cell. There is therefore no doubt that these di- and tri-carboxylic acids penetrate into the frozen cells. There is no evidence that monocarboxylic acids cannot penetrate into untreated yeast cells.

Fumarase has previously been demonstrated in extracts of brewer's yeast by Jacobsohn (1931) and aconitase in extracts of baker's yeast by Weinhouse & Millington (1947) and by Foulkes (1951). Thus under certain conditions the two enzymes can be extracted from the cells.

Variations in freezing procedure. The effects of freezing are not due to exposure to low temperature itself, but depend on the rate at which cooling takes place. If cells are gradually cooled by transfer first to a refrigerator (to 2°), then to a deep freeze (about −10°) and, when frozen solid, to dry ice, cells are obtained which on thawing are almost indistinguishable from untreated cells. Cooling in dry ice affects yeast cells only if it occurs rapidly, and the effects of freezing are the greater the more sudden the temperature drop near freezing point.

The oxidation of acetate and succinate by samples of yeast exposed to different treatments is shown in Table 2. Sample 1 was the untreated control. Sample 2, before mixing with dry ice, was kept for 2 hr. in a refrigerator (about +2°) and transferred to a deep freeze (−10°) where it was left for 4 hr. Sample 3 was kept for 3 hr. at room temperature and sample 4 was kept overnight in the refrigerator before dry-ice treatment. Samples 2–4 in addition were kept for 20 min. with an excess of dry ice, allowed to thaw and suspended in 39 vol. 0·1 M-phosphate buffer, pH 6·5, like the untreated sample. The manometer cups contained 3 ml. yeast suspension and 1 ml. 0·1 M-substrate solution. It will be

seen (Table 2) that transfer from room temperature of dry ice destroys almost completely the oxidation of acetate whilst it enhances that of succinate. Transfer from +2° to dry ice partly preserves the oxidation of acetate and increases the oxidation of succinate.

Table 2. *Effect of cooling procedure on the properties of frozen yeast cells*

(For treatment of samples see text; the O_2-uptake figures refer to the 80–100 min. period after addition of substrate.)

Sample no.	O_2 absorbed by 4 ml. yeast suspensions in 20 min. (μl.) Blank	Acetate	Succinate
1	44	458	48
2	59	388	62
3	66	75	113
4	66	211	97

In further experiments samples of yeast were kept for 1 hr. at 60, 50, 40 and 30°, respectively, before the dry-ice treatment, washed with 10 vol. of water and suspended in 19 vol. 0·1 M-phosphate buffer, pH 6·5. None of these samples oxidized acetate at an appreciable rate. The blank respiration and the oxidation of succinate are shown in Table 3. Exposure to 60° destroys most of the oxidizing capacity. Exposure to 50° abolishes the blank respiration almost completely but leaves the activity of the succinic-oxidase system. Exposure to 40 or 30° reduced the blank and increased the rate of oxidation of succinate. Table 3 further shows that the

Table 3. *Effect of prewarming of yeast cells before dry-ice treatment*

(Baker's yeast was warmed for 60 min. to the temperature specified. O_2 uptake by 4 ml. yeast cells containing 150 mg. fresh wt. Period 40–60 min. after addition of substrate; substrate concentration 0·020 M.)

Kept 1 hr. at	O_2 absorbed in 20 min. (μl.) Heated only Blank	Heated only Succinate	Heated and frozen Blank	Heated and frozen Succinate
60°	5	11	2	10
50°	170	199	3	100
40°	191	198	51	126
30°	156	161	112	163

effects observed at 50, 40 and 30° are not due to the heat treatment only, but mainly to the effect of solid CO_2 on the pre-warmed material.

The enzyme system responsible for the oxidation of succinate thus differs from those dealing with the oxidation of acetate. Cells preheated to 50° and then frozen in dry ice lend themselves with advantage to the study of the oxidation of succinate owing to the inactivity of most of the other oxidizing systems.

Observations on the oxidation of succinate. Yeast cells, preheated to 50°, treated with dry ice, washed once with 10 vol. of water and suspended in 0·1 M-phosphate buffer, pH 6·5, were used for the following experiments, unless otherwise stated. Of the substrates listed in Table 1, only succinate and D- and L-lactate were appreciably oxidized, the rate of oxidation of all three substances being about the same. Citrate caused a slight increase, amounting to about 50% of the small blank. The effect of the succinate concentration on the rate of oxidation is shown by the following figures representing the amount of O_2 absorbed in 20 min. by 4 ml. yeast suspension containing 150 mg. fresh weight:

Succinate concentration (M)	O_2 absorbed 40–60 min. after addition of substrate (μl.)
0·125	149
0·0625	139
0·03125	123
0·015625	113
0·00781	71

The oxidation of succinate was inhibited by 0·002 M-hydrogen cyanide and by saturated octanol. No CO_2 was formed. For each molecule of O_2 used, 2 molecules of succinate disappeared and 2 molecules of fumarate + L-malate were formed. The product of oxidation was determined manometrically with *Lactobacillus arabinosus* (Korkes & Ochoa, 1948; Nossal, 1952). Reducing the pretreatment period from 60 to 30 min. made no difference, whilst heating for 120 min. slightly lowered the rate of succinate oxidation.

Lynen (1943) has already found that the oxidation of succinate in yeast is competitively inhibited by malonate. This was confirmed by the observation that the inhibition depends on the succinate concentration; for example, the inhibition by 0·0025 M-malonate was 70% at 0·0625 M-succinate and 96% at 0·00625 M-succinate. Whilst in mammalian muscle tissue a 50% inhibition is found when the concentration ratio [succinate]/[malonate] is about 10 (Krebs & Johnson, 1948), the yeast enzyme showed a 50% inhibition when the ratio was about 60, as illustrated by the experiment recorded in Table 4. The relations between enzyme activity and the concentrations of substrate and a competitive inhibitor may be expressed by the formula

$$\frac{1-n}{n} \times \frac{[\text{substrate}]}{[\text{inhibitor}]} = K,$$

where n is $\dfrac{\text{reaction rate with inhibitor}}{\text{reaction rate without inhibitor}}$ and the brackets indicate concentrations (Krebs & Johnson, 1948). This formula holds provided that virtually all

Table 4. *Inhibition of yeast succinic dehydrogenase by malonate*

(Baker's yeast heated to 50° for 60 min.; cooled by dry ice for 16 hr. Washed once with water. Suspended in 19 vol. 0·1 M-phosphate buffer, pH 6·5. Per vessel 3 ml. yeast suspension, 1 ml. substrate-inhibitor solution. For significance of n and K see text.)

Substrate concentration (M)		O_2 absorbed in 60 min. (μl.)			
Succinate	Malonate	Observed	Corrected for blank	n	K
0	0	15	—	—	—
0·0625	0	254	239	—	—
0·0625	0·005	57	42	0·176	59
0·0625	0·0025	81	66	0·276	65
0·0625	0·00125	117	102	0·426	68
0·0625	0·000625	167	152	0·636	57
0	0·005	13	—	—	—

the enzyme is present either in combination with the substrate or with the inhibitor, but is not present in an uncombined form. K represents the ratio $\frac{\text{substrate}}{\text{inhibitor}}$ giving 50% inhibition. It will be seen from Table 4 that K is about 60. The relative affinity of succinic dehydrogenase for malonate is thus considerably higher in yeast than in animal tissue.

The effect of pH on the oxidation of succinate is shown in Table 5. The pH values were measured electrometrically and were found to agree with those calculated from the pK values (pK_H for triethanolamine = 7·77; for diethanolamine = 8·88; Hall & Sprinkle, 1932). There is a broad optimum between pH 7·4 and 8·5. Preliminary experiments showed that di- and tri-ethanolamine as such do not affect the O_2 uptake as there was no difference in phosphate buffer and di- and tri-ethanolamine buffers at pH 7·8.

Ethanolamine buffers are generally useful in enzyme experiments because of their high solubility and the absence of inhibiting effects; together with monoethanolamine (pK_H 9·44) they cover the pH range between 7 and 10. Stock solutions of the bases can be conveniently freed of bound CO_2 by boiling them in an open beaker until the volume is reduced to about half.

Effect of dry-ice treatment on anaerobic reactions of baker's yeast. Dixon & Atkins (1913) have already shown that liquid-air-treated brewer's yeast can ferment sugars, and Lynen (1939) found that liquid-air-treated brewer's yeast, in contrast to fresh yeast (see also Smythe, 1938; Barron, Ardao & Hearon, 1950), readily decarboxylates pyruvate. Data on the anaerobic CO_2 production by baker's yeast exposed to dry ice are shown in Table 6. The capacity to ferment glucose decreased on cold-treatment, whilst the rate of CO_2 evolution from pyruvate and α-ketoglutarate increased. All effects were more marked when the yeast was preheated to 30 or 50° before the exposure to dry ice. In sample 4 (Table 6) (preheating to 50°) glucose was not fermented, and this type of preparation may be used as a carboxylase for the determination of pyruvic acid in place of Lebedev juice from brewer's yeast

Table 5. pH-*Activity curve of the oxidation of succinate by cold-treated baker's yeasts*

(Yeast kept 60 min. at 50°, then treated with dry-ice. Washed once with water, resuspended in 19 vol. 0·1 M-buffer. Each vessel contained 3 ml. yeast suspension in the main compartment and 1 ml. 0·1 M-sodium succinate in the side arm; temp., 25°; gas, O_2. The data refer to O_2 absorbed for 10–40 min. period after addition of substrate. 'Salt' in the case of phosphate refers to Na_2HPO_4, and in the case of the amines to the hydrochlorides.)

Buffer	Ratio of salt to acid or base	pH	O_2 absorbed in 30 min. (μl.)
Acetate	4:1	5·3	18
Acetate	10:1	5·7	32
Phosphate	1:10	5·8	43
Phosphate	1:4	6·2	59
Phosphate	1:1	6·8	85
Phosphate	4:1	7·4	125
Phosphate	10:1	7·8	128
Triethanolamine	1:4	8·4	126
Diethanolamine	2:1	8·5	115
Diethanolamine	1:1	8·8	92
Diethanolamine	1:4	9·4	62

Table 6. *Anaerobic* CO_2 *production of cold-treated yeast*

(Baker's yeast suspended in 40 vol. 0·1 M-KH_2PO_4; 0·04 M-substrate; gas, N_2. Sample 1: untreated yeast; sample 2: transferred from refrigerator to dry ice; sample 3: left 60 min. at 30° before treated in the dry ice; sample 4: kept 60 min. at 50° before treated in the dry ice.)

Substrate	CO_2 formed in 30 min. (μl.)			
	Sample 1	Sample 2	Sample 3	Sample 4
None	8	8	2	2
Glucose	1860	730	368	4
Pyruvate	15	244	422	120
α-Ketoglutarate	10	21	30	10

(Krebs & Johnson, 1937). A sample (1 ml.) of a yeast suspension in 0·1 M-acetate buffer, pH 4·5, containing 0·5 g. yeast decomposed 0·1 ml. 0·2 M-pyruvate in 0·05 M-acetate buffer, pH 4·5, within 40 min. at 25°.

Effect of malonate on the oxidation of acetate

As in mammalian tissues, malonate can be used in frozen yeast cells to test the participation of succinic dehydrogenase in the oxidation of acetate. For this test cells are required which are capable of oxidizing both succinate and acetate. Yeast transferred from the refrigerator to dry ice and, after washing, suspended in phosphate buffer, pH 6·5, was therefore used. Further details of a representative experiment are recorded in Table 7. The yeast used had a considerable blank respiration; for the interpretation of the results this has been subtracted from the values observed on addition of substrates. Whilst the oxidation of succinate was almost completely inhibited by malonate there was no effect on the oxidation of acetate or on the blank respiration. The effect on the oxidation of succinate indicates that malonate freely penetrated into the cells. The results lead to the conclusion that under the test conditions succinate could not be an intermediate in the oxidation of acetate or the blank respiration, unless the improbable assumption is made that the oxidation of succinate and acetate takes place at different sites and that malonate has access to the site of succinate oxidation, but not to that of acetate oxidation.

These experiments are in apparent contradiction to recent results of Barron *et al.* (1950) who found an inhibition by malonic acid of the oxidation of pyruvic acid in acid media (pH 2·8), and also to those of Lynen (1943) who observed a malonic acid inhibition of the oxidation of acetic acid in acid media. The experiment recorded in Table 8 confirms the findings of Barron *et al.* and Lynen, including the high rate of oxidation of pyruvic and acetic acids in the acid medium. It is noteworthy that relatively high concentrations (0·0125 M) of malonic acid are required; there was no inhibition at 0·001 M-malonic acid. Added fumaric, succinic and citric acids are not appreciably oxidized by cells suspended in the acid medium, nor do these substrates affect the malonic acid inhibition of pyruvic or acetic acid oxidation. Lynen (1943), in contrast, found a partial reversal by fumarate of the malonate inhibition of acetic acid oxidation.

A further study of the malonic acid effect showed that its characteristics are very different from those of the inhibition of succinic dehydrogenase. Firstly, the inhibition is irreversible. After incubation for 1 hr. the cells were centrifuged, washed once in phosphate buffer, and resuspended in the original volume. The rate of respiration with pyruvic acid

Table 7. *Effect of malonate on the oxidation of acetate*

(Dry-ice-treated baker's yeast (2 g.) shaken 5 min. in 80 ml. 0·125 M-phosphate buffer, pH 6·5; centrifuged, resuspended in 40 ml. buffer. Per cup 3 ml. ≡ 150 mg. fresh cells. Added substrate and malonate were contained in 1 ml.; temp., 25°; gas., O_2. The concentrations refer to final values.

Arrangement and O_2 consumption									
Substrate(s) added	Nil	Succinate (0·025 M)	Succinate (0·025 M) + malonate (0·025 M)	Succinate (0·025 M) + malonate (0·005 M)	Succinate (0·025 M) + malonate (0·001 M)	Malonate (0·025 M)	Acetate (0·025 M)	Acetate (0·025 M) + malonate (0·025 M)	Acetate (0·025 M) + malonate (0·005 M)
Absorbed O_2 (μl.):									
20–30 min	316	414	334	335	376	318	630	577	620
60–100 min.	132	210	137	128	173	139	658	596	646
Malonate inhibition (%) during time 60–100 min.	—	—	90*	100*	40*	—	—	9†	2†

* Percentage of O_2 uptake due to addition of substrate.

† Percentage of total O_2 uptake.

was the same as before washing with pyruvic and malonic acids. Secondly, no accummulation of succinic acid in the presence of malonic acid could be demonstrated, in contrast to the findings of Barron *et al.* (1950) and of Lynen (1943). The experimental conditions were similar to those described by Barron *et al.* (1950) (see especially Table IV, p. 220) but 0·1 M-phosphoric acid buffer, pH 2·2, consisting of one part of phosphoric acid and two parts of potassium dihydrogen phosphate was used as a medium. The suspension contained 180 mg. fresh yeast in 4 ml. The concentration of pyruvic acid was

Table 8. *Malonate inhibition of yeast respiration in acid medium*

(Yeast suspension (3 ml.): 1·5 g. fresh baker's yeast in 100 ml. buffer (20 ml. 0·1 M-H_3PO_4, 80 ml. 0·1 M-KH_2PO_4, pH 2·5). 1 ml. substrate and/or inhibitor solution. Substrates added as free acids to final concentration 0·0125 M; temp., 25°; gas, O_2.)

Substrates added	O_2 absorbed in 30 min. (μl.)
None	29
Pyruvic acid	515
Acetic acid	333
Citric acid	30
Succinic acid	35
Fumaric acid	33
Pyruvic and malonic acids	95
Acetic and malonic acids	46
Acetic, malonic and fumaric acids	49
Pyruvic and fumaric acids	510
Pyruvic, malonic and fumaric acids	90
Glucose	520
Glucose, malonic acid	140
Glucose, malonic and fumaric acids	158

0·025 M, of malonic acid 0·0125 M. The maximum Q_{O_2} in this medium in the presence of pyruvate was 80 μl./mg. dry wt./hr. After 110 min. incubation the total O_2 consumption with pyruvic acid was 4120 μl., and in the presence of pyruvic acid and malonic acid 1430 μl. The solutions were deproteinized with tungstic acid and the filtrate was treated with acid permanganate (see Krebs & Eggleston, 1948). No appreciable quantities of succinic acid were found, whilst succinic acid added to the yeast suspensions and incubated for the same period was almost quantitatively recovered. It is, however, confirmed that under the conditions used by Lynen (1943, Table 4, p. 54) succinate is formed aerobically in the presence of fumaric, acetic and malonic acids. Lynen used very high yeast concentrations (16·7 % wet wt. in the incubated suspensions) and the rate of respiration was very low in his experiment (Q_{O_2} about -6). No appreciable amounts of succinate were found in more dilute yeast suspensions, e.g. in the experiment recorded in Table 8.

Thirdly, the inhibition by malonic acid is not specific; similar effects are given by oxalic acid, equal concentrations of oxalic and malonic acids being about equally effective. In contrast, yeast succinic dehydrogenase under the conditions specified in Table 4 was not inhibited by 0·01 M-oxalate. Fourthly, the inhibition by malonic acid was independent of the concentration of added substrates (succinic, pyruvic or acetic acids) and was thus not competitive.

In view of these findings the malonic acid inhibition of fresh yeast respiration in a strongly acid medium cannot be taken as evidence of a participation of succinic dehydrogenase. The mechanism of this inhibition remains to be elucidated.

It is remarkable that fresh yeast cells show a normal rate of respiration in a medium of pH 2·1. Measurement of the intracellular pH indicates that this was not affected by the acidity of the medium. For the measurement of the intracellular pH the yeast suspension after incubation with pyruvic acid was centrifuged in a plastic centrifuge tube, the supernatant was poured off and the residue washed once in a small volume of water. This was decanted and the tube placed in dry ice, to remove permeability barriers. After thawing, the pH was measured electrometrically in the liquefied material. A pH value of 5·7 was obtained.

Experiments with [^{14}C] *acetate*

Isotopic acetate together with non-isotopic succinate, fumarate, malate, or α-ketoglutarate, was added to yeast cells which had been made permeable to di- and tri-carboxylic acids by cold-treatment. Yeast was transferred from the refrigerator (+2°) to dry ice, washed twice with water and suspended in 24 vol. of 0·1 M-phosphate buffer, pH 6·5. In order to provide sufficient material for the isolation and quantitative determination of intermediates, large conical Warburg vessels of about 120 ml. capacity were used (see Krebs & Eggleston, 1945). In most experiments the flask contained 20 ml. yeast suspension and 5 ml. additional substrate solution. The centre well contained 1 ml. CO_2-free 2 N-sodium hydroxide and filter paper. In most experiments the O_2 uptake during the period of incubation was measured. The term 'extra O_2' in the following tables refers to the additional O_2 consumption caused by the substrate. At the end of the experiment the filter paper in the centre well was removed, rinsed with water and, together with the alkali solution, transferred to a centrifuge tube containing a solution of barium hydroxide. The barium carbonate was centrifuged, washed and dried for the radioactivity assay.

Isotopic material. Carboxyl-labelled sodium acetate was prepared from non-radioactive methyl iodide and radioactive CO_2 by the Grignard reaction according to Sakami, Evans & Gurin (1947). The radioactive acetate was diluted with carrier acetate to give 1580 counts/min./mg. carbon.

Methyl-labelled sodium acetate was obtained from Tracerlab Inc., Boston. After dilution with carrier acetate it gave 921 counts/min./mg. carbon.

Radioactivity measurements. All measurements were made with a mica end-window counter on samples of $BaCO_3$ obtained by wet oxidation of the organic substances according to Van Slyke & Folch (1940). Plates of $BaCO_3$ were prepared by grinding the $BaCO_3$ with acetone, pouring the resulting suspension quickly on to an aluminium disk fitted with a stainless-steel cylinder and evaporating the acetone by radiation from a lamp placed above the cylinder. The weight of the $BaCO_3$ layer was determined and corrections for self-absorption were applied in order to obtain the activity at infinite thickness (20 mg. $BaCO_3$/cm.^{2}) using the correction table of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The counting time was sufficiently long so that the standard error was no greater than 5%.

precipitated with a solution of $HgNO_3$ (10% (w/v)) in 5% (v/v) HNO_3 (Stotz, 1937). The precipitate was centrifuged and reprecipitated by dissolving it in 15% (v/v) HNO_3 at 100°, adding 2 vol. of water and the $HgNO_3$ reagent. A nearly colourless precipitate, obtained on chilling, was washed in water, suspended in water and treated with H_2S. The colourless filtrate was evaporated to 1–2 ml. and chilled. Crystals of fumaric acid appeared which, after two recrystallizations from hot water and drying *in vacuo*, sublimed at 200° and melted at 285–290°.

For the isolation of malic acid the filtrates of the $HgNO_3$ precipitation were continuously extracted with ether for 5 hr. in the Kutscher-Steudel apparatus. The ether was evaporated and the residue taken up in a small volume of water. The solution was acidified with HNO_3 to pH 1, mixed with 1 ml. 10% (w/v) $AgNO_3$ and brought to pH 6 with 0·5 N-ammonia. The silver salts were centrifuged off, re-

Table 9. *Simultaneous oxidation of* $CH_3.{}^{14}COOH$ *and non-isotopic succinate*

(For general conditions see text; incubation 110 min.; flask 4 was duplicated to supply sufficient material.)

Arrangement and O_2 consumption

Flask	1	2	3	4
Substrate	None	Succinate (0·02 M)	$CH_3.COOH$ (0·02 M)	$CH_3.{}^{14}COOH$ (0·02 M) + succinate (0·02 M)
O_2 absorbed (μl.)	5 600	11 110	13 570	18 120
Extra O_2 (μl.)	—	+5 510	+7 970	+12 520

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0·75	1580
Acetic acid, final	0·44	1572
CO_2, final	—	627
Fumaric acid	—	6
Malic acid	—	3
Succinic acid, initial	0·50	0
Succinic acid, final	Nil	—

Simultaneous oxidation of carboxyl-labelled acetate and succinate. The arrangement of the experiment and data on the O_2 consumption are shown in Table 9. It will be seen that both succinate and acetate increased the O_2 consumption and that the effects were additive. At the end of the incubation period the contents of the duplicate flasks 4 were combined. The determination of succinate in a 3 ml. sample showed that all added succinate had been oxidized. The remaining suspension was deproteinized with 5 ml. 15% HPO_3, and acetic acid was separated by steam distillation. The distillate was titrated with standard alkali and evaporated to dryness. The residue was repeatedly extracted with 3 ml. boiling 95% (v/v) ethanol. The combined extract was evaporated to a small volume and sodium acetate precipitated with ether. The precipitate was centrifuged, dried, dissolved in a little water and acidified to pH 1 with HNO_3. 10% (w/v) $AgNO_3$ (1 ml.) was added and the pH was adjusted to 5–6 with 0·5 N-ammonia. Silver acetate was centrifuged off, washed three times with a little cold water, once with ethanol, once with ether, and dried *in vacuo*. The specimen obtained served for the measurement of radioactivity.

The non-volatile residue was acidified with HNO_3 to a final concentration of 5% (v/v) and fumaric acid was precipitated with a solution of $HgNO_3$ (10% (w/v)) in 5% (v/v) HNO_3 (Stotz, 1937). The precipitate was centrifuged and reprecipitated by dissolving it in 15% (v/v) HNO_3 at 100°, adding 2 vol. of water and the $HgNO_3$ reagent. A nearly colourless precipitate, obtained on chilling, was washed in water, suspended in water and treated with H_2S. The colourless filtrate was evaporated to 1–2 ml. and chilled. Crystals of fumaric acid appeared which, after two recrystallizations from hot water and drying *in vacuo*, sublimed at 200° and melted at 285–290°.

For the isolation of malic acid the filtrates of the $HgNO_3$ precipitation were continuously extracted with ether for 5 hr. in the Kutscher-Steudel apparatus. The ether was evaporated and the residue taken up in a small volume of water. The solution was acidified with HNO_3 to pH 1, mixed with 1 ml. 10% (w/v) $AgNO_3$ and brought to pH 6 with 0·5 N-ammonia. The silver salts were centrifuged off, dissolved in 0·1 N-HNO_3 and reprecipitated with NH_4OH at pH 6. The precipitate obtained was treated in aqueous suspension with H_2S and filtered off. The filtrate was evaporated to dryness, the residue dissolved in about 2 ml. warm ethanol and neutralized by the addition of solid brucine. On chilling, dibrucine malate separated out. It was recrystallized three times from a small quantity of boiling ethanol; m.p. and mixed m.p. 207–208°. The dibrucine salt was dissolved in a little water, made alkaline with 2 N-NaOH and the suspension was extracted three times with ether to remove brucine. The aqueous residue was acidified to pH 1 with HNO_3 and silver malate was obtained by addition of $AgNO_3$ and adjustment of pH to 6. The silver salt was centrifuged and reprecipitated from dil. HNO_3. It was finally washed with water and dried *in vacuo* for radioactivity measurements.

The results of the radioactivity measurements are shown in Table 9. The specific activity of the recovered acetate was the same as that of the added acetate showing that no new acetate had been formed. The CO_2 formed was radioactive, showing that the acetate removed had been oxidized to CO_2.

Some of the added succinate was recovered as fumarate and malate, both acids being inactive.

The experiment proves that fumarate and malate cannot have been intermediates in the oxidation of acetate. Any fumarate and malate that might have arisen from acetate by the di- or tricarboxylic acid cycles would have been radioactive and would have mixed with the fumarate and malate arising from succinate. The recovered fumarate and malate would therefore have been radioactive. The inactivity of the acids cannot have been due to permeability barriers because the substrate arose from succinate within the cells.

As all the added succinate had been removed, a shorter period of incubation was chosen in a second experiment with the same substrates. Because of the shorter incubation period, less acetate was added. Details of the experiment are given in Table 10. The quantitative manometric succinate determination at the end of the incubation showed that about half of the added quantity had been removed. No succinate was found in flasks 1 and 3.

Succinic acid was recovered from the residue of the deproteinized filtrate after the removal of acetic acid by steam distillation. The residue was concentrated *in vacuo* to 40 ml. and continuously extracted with ether for 90 min. After evaporation of ether the residue was dissolved in 4 ml. hot ethyl acetate. The solution was filtered and the filtrate concentrated to 1 ml. by a current of dry air. The precipitate obtained on chilling was recrystallized twice from ether; m.p. and mixed m.p. 190°.

Whilst the CO_2 was radioactive the recovered succinate was inactive (Table 10). Thus succinate cannot have been formed by the oxidation of acetate.

Simultaneous oxidation of methyl-labelled acetate and succinate. The general procedure was the same as in the preceding experiments. Details are given in Table 11. It will be seen that 18% of the added acetate and 78% of the added succinate were removed. The CO_2 formed was radioactive, whereas

Table 10. *Simultaneous oxidation of* $CH_3.{}^{14}COOH$ *and non-isotopic succinate*

(Incubation 57 min.; for other conditions see Table 9.)

	Arrangement and O consumption			
Flask	1	2	3	4
Substrate	None	Succinate (0·02 M)	$CH_3.COOH$ (0·0092 M)	Succinate (0·02 M) + $CH_3.{}^{14}COOH$ (0·0092 M)
O_2 used by 25 ml. suspension (μl.)	3860	4560	6210	7280
Extra O_2 (μl.)	—	+700	+2350	+3420

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0·23	1580
Acetic acid, final	0·12	—
Succinic acid, initial	0·50	0
Succinic acid, final	0·29	3
CO_2, final	—	485

Table 11. *Simultaneous oxidation of* $^{14}CH_3.COOH$ *and succinate*

(For general conditions see text; incubation 80 min.)

	Arrangement and O_2 consumption			
Flask	1	2	3	4
Substrate	None	Succinate (0·02 M)	$CH_3.COOH$ (0·02 M)	$^{14}CH_3.COOH$ (0·02 M) + succinate (0·02 M)
O_2 used by 25 ml. suspension (μl.) ...	2400	4360	4290	6190
Extra O_2 (μl.)	—	+1960	+1890	+3790

Substrate changes and isotope data in flask 4

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0·50	921
Acetic acid, final	0·41	—
Succinic acid, initial	0·50	0
Succinic acid, final	0·11	0·4
CO_2, final	0·27	188

the recovered succinate was inactive. The experiment thus confirms those with carboxyl-labelled acetate.

Simultaneous oxidation of carboxyl-labelled acetate and α-ketoglutarate. Isotope data on an experiment with α-ketoglutarate are shown in Table 12.

After steam distillation of the deproteinized yeast filtrate the solution containing the non-volatile substances was concentrated *in vacuo* to about 25 ml. and continuously extracted with ether for 2 hr. The ether was evaporated to dryness, the residue taken up in 2 ml. water, and the α-ketoglutaric acid semicarbazone precipitated by the addition of solid semicarbazide hydrochloride. After chilling, the crystalline material was centrifuged and recrystallized three times from hot water; m.p. and mixed m.p. 200°.

Table 12. *Simultaneous oxidation of* $CH_3.{}^{14}COOH$ *and α-ketoglutarate*

(For general conditions see text; incubation 120 min.; 25 μl. yeast suspension.)

Substance	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0·50	1580
Acetic acid, final	0·40	—
α-Ketoglutaric acid, initial	0·50	0
α-Ketoglutaric acid, final	—	0·6
CO_2, final	0·27	700

α-Ketoglutarate did not contain ^{14}C (Table 12) and is thus excluded as an intermediate in the oxidation of acetate.

Simultaneous oxidation of formate and doubly labelled acetate. A mixture of carboxyl- and methyl-labelled acetate and fresh yeast (not treated with dry ice) were used; otherwise the experimental conditions were similar to those of the preceding experiments. To isolate formic acid the deproteinized filtrate was steam distilled. The distillate was treated with $HgSO_4$ in H_2SO_4 solution and the CO_2 evolved from formic acid was collected as $BaCO_3$. Acetic acid was recovered by steam distillation from the solution after the removal of formic acid and isolated as the Ag salt as previously described.

Formic acid was found to be inactive (Table 13).

Table 13. *Simultaneous oxidation of* $^{14}CH_3.{}^{14}COOH$ *and formate*

(For general conditions see text; incubation 60 min.; 25 ml. yeast suspension.)

Substance	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic, acid initial	0·20	1250
Formic acid, final	—	4
CO_2, final	—	488

Simultaneous oxidation of glyoxylic acid and methyl-labelled acetate. Dry-ice-treated yeast was used. At the end of the incubation period of 60 min. the suspension was mixed with 2 vol. of ethanol and centrifuged after storage in the cold. The clear supernatant was concentrated *in vacuo* to a few ml., freed of insoluble material by centrifugation and mixed with solid semicarbazide hydrochloride.

The colourless crystals of glyoxylic acid semicarbazone did not contain ^{14}C.

Simultaneous oxidation of acetoacetate and carboxyl-labelled acetate. The incubated yeast suspension was treated with 5 ml. 20% (w/v) $CuSO_4$ and 5 ml. 10% (w/v) $Ca(OH)_2$ suspension. The filtrate was acidified to pH 3 and aerated for 15 min. at room temperature. Denigès mercuric sulphate reagent was added and the CO_2 formed on heating under reflux and derived from the carboxyl group of acetoacetate was collected. The mercury-acetone precipitate was centrifuged, redissolved in 2 N-HCl and the acetone was distilled into fresh Denigès reagent. The distillate was refluxed with Denigès reagent and the precipitate was centrifuged, washed and dried.

Both the CO_2 and the acetone derived from the acetoacetate were inactive (Table 14).

Table 14. *Simultaneous oxidation of* $CH_3.{}^{14}COOH$ *and acetoacetate*

(For general conditions see text; incubation 60 min.; 25 ml. suspension fresh yeast.)

Substance	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	0·20	1580
Acetoacetic acid	0·20	—
CO_2 derived from acetoacetic acid	—	2
Acetone derived from acetoacetic acid	—	0
CO_2, final	—	831

Homogeneity of cold-treated yeast

Dr Sidney Weinhouse has personally pointed out to the authors that the results of the simultaneous oxidation experiments, as well as those on the malonate inhibition, would be inconclusive if suspensions of cold-treated yeast cells contained two different types of cells: (1) normal cells capable of oxidizing acetate and incapable of oxidizing added succinate, this incapacity being due to a permeability barrier; (2) 'damaged' or 'dead' cells incapable of oxidizing acetate and capable of oxidizing added succinate; the prototype of such damaged cells are those heated to 50° and then exposed to dry ice as described earlier in this paper. If this kind of inhomogeneity existed, the oxidation of succinate and acetate in the cold-treated yeast suspension would occur at different sites and the results reported in this paper could not be regarded as evidence against the participation of the dicarboxylic acids in the oxidation of acetate.

Attempts to detect two types of cells by vital staining with methylene blue or triphenyltetrazolium chloride remained inconclusive, but the following two experiments meet the criticism.

In the first experiment the specific activity of intra- and extra-cellular succinic acid after incubation of fresh yeast cells with $^{14}CH_3.COOH$ was measured. The type of baker's yeast used contained 2–3 mg. intracellular succinic acid/g. fresh weight. Some of this was lost to the medium on incubation and in order to obtain enough cell material for the isolation of succinic acid a relatively high concentration of cells was used. The temperature was kept low as this makes it easier to saturate the concentrated cell suspension with O_2.

Fresh baker's yeast (2·5 g.) was suspended in 22·5 ml. of medium containing methyl-labelled acetate buffer (0·0316 M), pH 5·0, and unlabelled succinate (0·00236 M), and shaken in large conical Warburg vessels for 100 min. in O_2 at 8°. Preliminary experiments had shown that under these conditions the intracellular concentration of succinate remains above that of the medium. Duplicate flasks were set up in order to provide sufficient material for the isolation of succinic acid. The respiratory CO_2 was absorbed by 2 N NaOH contained in the centre well.

On completion of the incubation period the cell suspension was ice-cooled and centrifuged. The supernatant was decanted and stored at −14° until it could be analysed. The sediment was diluted with water to 20 ml. and deproteinized with 5 ml. 15 % HPO_3. The precipitate was discarded. The decanted supernatant medium and the deproteinized extract of the cells were treated in the same way. Acetic acid was distilled off at pH 2 and titrated with NaOH in the distillate. Succinic acid was extracted from the residue with ether, dissolved in water and determined in one-fifth of the volume. The remaining succinate was diluted with carrier succinic acid before further recrystallizations. A sample (5·3 mg.) of succinic acid obtained from the cells was diluted with 15·4 mg. carrier, and 18·3 mg. carrier were added to 7·1 mg. obtained from the medium. In all other respects the procedures were as before. The relevant data are given in Table 15.

About half of the added acetate was removed during incubation. The respiratory CO_2 was radioactive, but its specific activity was only about 3 % of that of the acetate. Differences between the specific activities of CO_2 and acetic acid may be partly due to the high blank respiration which was 77·5 % of that of the sample containing acetate. It is also probable that the unlabelled carboxyl carbon of acetate yields CO_2 more rapidly than the methyl carbon. If acetate were oxidized through the tricarboxylic acid cycle the first two turns of the cycle would yield four unlabelled and no labelled CO_2 molecules. Only during the third turn would labelled CO_2 begin to appear. Similar relations may hold for other intermediary mechanisms.

The intracellular succinate was highly radioactive, the specific activity being about one-quarter of that of the acetate. This result is in accordance with that of Lynen (1947) who found deuterosuccinate on incubation of baker's yeast with fumarate and deuteroacetate. But the most significant finding was the radioactivity of the extracellular succinic acid which was almost twice that of the respiratory CO_2. It shows that fresh cells are in fact not completely impermeable to succinate and can discharge the substance into the medium. This is also borne out by the increase in the concentration of succinate in the external medium. The differences in the specific activity of the extra- and intra-cellular succinic acid, however, indicate that the permeability is restricted.

Table 15. *Specific activity of intra- and extracellular succinic acid after incubation of fresh yeast with* $^{14}CH_3.COOH$

(For general conditions see text; the data refer to 50 ml. suspension.)

Arrangement and O_2 consumption

Flask no.	1	2
Substrate	Succinate (0·00236 M)	Succinate (0·00236 M) + acetate (0·0316 M)
O_2 absorbed (μl.)	14 610	18 870

Substrate changes and isotope data in flask 2

	Amount (m-moles)	Specific activity (counts/min./mg. C)
Acetic acid, initial	1·59	6195
Acetic acid, final	0·930	—
CO_2, final	—	193
Succinic acid, initial, intracellular	0·110	0
Succinic acid, initial, extracellular	0·118	0
Succinic acid, initial, total	0·228	—
Succinic acid, final, intracellular	0·049	1520
Succinic acid, final, extracellular	0·256	344
Succinic acid, final, total	0·305	—

Before the significance of these results is further considered it should be mentioned that the experiment was repeated with washed cold-treated cells.

The conditions were the same except that the initial succinate concentration was higher and equal to that of acetate. This was necessary because of the rapid oxidation of succinate in cold-treated cells; it also favoured the trapping of labelled succinate. The experiment differed from that recorded in Table 11 in respect to pH, temperature and especially a much higher specific activity of the added acetate (6195 counts/min./mg. C) which again favoured the detection of a formation of succinate from acetate. Cells and medium were not separated for the analysis.

The specific activity of the respiratory CO_2 was found to be 1920 counts/min./mg. C and that of the succinic acid isolated at the end of incubation was 34. This value is well above the limits of error, but it is far too low to justify the conclusion that an appreciable part of the oxidized acetate had passed through the stage of succinate. It should be recalled that if mixing between the succinate formed as an intermediate and that present in the medium is

complete the specific activity of succinate must be higher than that of the respiratory CO_2.

The conclusion may be drawn from these experiments that cold-treated yeast contained no appreciable quantities of 'intact' cells behaving like fresh yeast; otherwise radioactive succinate would have been found under the test conditions. They also show that cold-treatment almost completely destroys the mechanism whereby succinic acid is produced from acetate, whilst that responsible for the oxidation of acetate to CO_2 remains.

The second experiment was designed to assess quantitatively the proportion of cells in a dry-ice-treated sample which, like fresh cells, possesses a permeability barrier for dicarboxylic acids. It is based on the fact that fresh cells maintain a high concentration of L-malate within the cells when shaken in aqueous media containing no L-malate, whilst 'damaged' cells no longer maintain concentration gradients.

Fresh cells and cold-treated cells (2·5 g.) were vigorously shaken for 15 min. at 10° in 47·5 ml. of water. A measured sample of this suspension (48 ml.) was then quickly ice-cooled and centrifuged. After decanting, the volume of the supernatant was measured and reduced on a steam bath to about one-quarter. The volume of the sediment was available by difference and the amounts of cells and supernatant in the residue were calculated assuming the specific gravity to be 1·090. The total residue was diluted with water to a volume of 10 ml. and heated for 15 min. to 100°. Malate was determined in 2·5 ml. samples of this suspension by the method of Nossal (1952) and in the concentrated supernatant.

The results are shown in Table 16. Fresh cells contained 8·44 μmoles malate/g. after shaking.

Table 16. *Comparison of the distribution of* L-*malate between cells and medium in fresh and dry-ice-treated baker's yeast*

(The data refer to 2·5 g. of yeast suspended in 47·5 ml. of water. For further details see text.)

Treatment of cells	L-Malic acid found (μmoles)			
	Cells		Supernatant	
	In total	Per g.	In total	Per ml.
Untreated	21·1	8·44	1·98	0·042
Dry-ice-treated	3·61	1·44	23·8	0·50

Only about 10 % of the intracellular malate had been lost to the medium and the concentration in the medium was about $\frac{1}{500}$th of that in the cells. The total malate content of the suspension of cold-treated cells was higher than that of the fresh cells, because cold-treated cells oxidize intracellular succinate to fumarate and malate, but over 85 % of the malate was present in the medium. The concentration in the cells was still a little higher than in the medium, and on the assumption that the excess found in the cells was due to an admixture of 'undamaged' cells it can be calculated that 7 % of the cells were undamaged. This is a maximum value because some of the intracellular malate may have been formed by oxidation of succinate during and after centrifugation. Even ice cooling and rapid manipulation in the analysis of the residue cannot completely eliminate this source of error. It is noteworthy that succinate leaves fresh cells much more readily than L-malate. Under identical conditions about half the intracellular succinate was lost in the medium.

DISCUSSION

Terminal oxidation mechanisms in yeast. Two independent sets of observations provide evidence against the view that the tricarboxylic acid cycle or a dicarboxylic acid cycle is the main terminal oxidation mechanism in dry-ice-treated yeast. The fact that malonate has no effect on the oxidation of acetate under conditions where it blocks oxidation of succinate alone is proof against the participation of succinate in the oxidation of acetate. The simultaneous-oxidation experiments with isotopic acetate and non-isotopic additional substrate are also proof of the non-participation of succinate, fumarate, malate or α-ketoglutarate, as well as of glyoxalate or formate, as intermediates in the oxidation of acetate.

The failure to find C_2 or C_1 compounds that fulfil the requirements of intermediates in the oxidation of acetate suggests that acetate, in order to undergo oxidation, must enter some kind of condensation reaction. One of the possibilities which suggests itself, considering the relative stability of acetylcoenzyme A (Lynen & Reichert, 1951), is the oxidation of acetate in the form of acetylcoenzyme A, the intermediate stages being glycollyl-, glyoxalyl-, oxalyl- and formyl-coenzyme A. That coenzyme A is required for the oxidation of acetate in yeast is suggested by the observation of Novelli & Lipmann (1947) that the rate of oxidation of acetate is reduced in coenzyme A-deficient yeast cells.

Occurrence of the component reactions of the tricarboxylic acid cycle. Qualitatively all the component reactions of the tricarboxylic acid cycle have been shown to occur in yeast cells. The relevant facts may be summarized as follows.

(1) Citric acid can be formed by yeast cells when magnesium or barium acetate is added (Wieland & Sonderhoff, 1932; Virtanen & Sundman, 1942; Weinhouse & Millington, 1947). Citric acid is formed in extracts from yeast cells when acetate and oxaloacetate are present (Novelli & Lipmann, 1950).

(2) Citric acid can be converted to α-ketoglutaric acid by preparations of baker's yeast and of pure cultures of *Saccharomyces cerevisiae* (Foulkes, 1951; Kornberg & Pricer, 1951).

(3) Preparations of yeast cells can oxidize succinate to fumarate and malate to oxaloacetate under certain conditions, as was first shown by Lynen (1943).

(4) Yeast extracts contain fumarase (Jacobsohn, 1931) and aconitase (Weinhouse & Millington, 1947; Racker, 1950; Foulkes, 1951).

Thus all the enzyme systems required for the tricarboxylic acid cycle are present in yeast cells, but with the exception of fumarase and aconitase the activities of the enzymes are low in comparison with the rate of acetate oxidation, the slowest reaction being the oxidation of malate to oxaloacetate.

The role of the tricarboxylic acid cycle in yeast cells. If the cycle is not the mechanism by which energy is produced from acetate, but if the enzyme systems responsible for its component reactions nevertheless occur in the cell, the problem of the physiological significance of the cycle presents itself. It is relevant with regard to this problem that in addition to the energy-giving mechanism, there is another major set of chemical changes in rapidly growing organisms, namely, the synthetic processes connected with growth. As far as the turnover of carbon is concerned both types of reactions can be of the same order of magnitude (see Clifton, 1946). The observation that resting yeast cells accumulate succinate when glucose is available, whilst growing cells do not (Kleinzeller, 1941) and that growing cells, in contrast to resting cells, can utilize added succinate, fumarate and malate at low pH values (Nossal, 1951) supports the assumption that the tricarboxylic acid cycle is related to growth in yeast cells. The findings of Ehrensvärd, Reio, Saluste & Stjernholm (1951), who studied the synthesis of amino-acids in *Torulopsis utilis* grown on a medium in which $^{13}CH_3.{}^{14}COOH$ was the only source of carbon, are in accordance with the view that the cycle supplies carbon skeletons for the synthesis of organic substances. At the same time these authors come to the conclusion that formation of citrate by condensation of acetate with oxaloacetate cannot be the main entrance of acetate into intermediary metabolism.

There are numerous observations, briefly summarized in the following section, which support the view that in many other (though not in all) micro-organisms the tricarboxylic acid cycle supplies not primarily energy but intermediates.

The role of the tricarboxylic acid cycle in other micro-organisms

Escherichia coli. Esch. coli fails to grow on media in which citrate is the sole source of carbon (Koser, 1923); yet if citrate is present in the medium together with glucose and a source of nitrogen, citrate is utilized (Lominski, Conway, Harper & Rennie, 1947). These observations may be interpreted as indicating that citrate cannot serve as a source of energy but may be used by growing cells for other purposes. The occurrence of the tricarboxylic acid cycle in *Esch. coli* is also made improbable by simultaneous-oxidation experiments of Ajl & Kamen (1951). Addition of labelled acetate and unlabelled succinate, α-ketoglutarate, fumarate, malate and oxaloacetate led to the incorporation of labelled carbon in the C_4 dicarboxylic acids, but not in α-ketoglutarate. Citrate was not tested. Whilst these data argue against the oxidation of acetate through the tricarboxylic acid cycle they permit the assumption of a dicarboxylic acid cycle of the oxidation of acetate. Experiments of Cutinelli, Ehrensvärd, Reio, Saluste & Stjernholm (1951) analogous to those quoted for *Torulopsis* (Ehrensvärd *et al.* 1951) directly support the assumption that the synthesis of the carbon skeleton of amino-acids, e.g. glutamic acid, aspartic acid and alanine, involves the reactions of the tricarboxylic acid cycle. Moreover, some of the necessary enzymes, namely those responsible for the synthesis of citric acid from oxaloacetate and acetate (Novelli & Lipmann, 1950; Stern & Ochoa, 1949), have been demonstrated.

Aerobacter aerogenes. Simultaneous-oxidation experiments with ^{14}C-labelled acetate and non-labelled intermediates of the tricarboxylic acid cycle on *A. aerogenes* grown on a citrate-containing medium showed labelling in all intermediates of the tricarboxylic acid cycle including citrate and α-ketoglutarate (Ajl & Wong, 1951). These findings suggest the occurrence of the tricarboxylic acid cycle in this organism, but further observations indicate that a second mechanism is concerned with acetate oxidation. The specific activity of the respiratory CO_2 was much higher than that of any carbon atom of citrate, α-ketoglutarate or succinate. This might be explained by one of two assumptions: either the added intermediates were not in equilibrium with the intracellular substances, or there is a second mechanism of acetate oxidation, in which the above intermediates do not take part. Other findings of Ajl & Wong (1951) are in favour of the second alternative. In the presence of an intermediate of the tricarboxylic acid cycle, the acetate recovered at the end of the incubation period had a lower specific activity than the starting material, and when inactive acetate but radioactive intermediates of the cycle were added the acetate became active on incubation. Thus the oxidation of the di- and tri-carboxylic acids leads to the formation of acetate, which is difficult to reconcile with the tricarboxylic acid cycle, but is expected for the dicarboxylic acid cycle.

'Simultaneous-adaptation' experiments (Stanier, 1947) likewise argue against the participation of citrate in the main oxidation mechanism. Citrate-adapted cells are also adapted to oxidize acetate, but

the reverse is not true; acetate-adapted cells do not oxidize citrate rapidly.

Micrococcus lysodeikticus. Simultaneous-oxidation experiments by Ajl, Kamen, Ranson & Wong (1951) indicate that in this organism α-ketoglutarate and the C_4 dicarboxylic acids participate equally as intermediates in the oxidation of acetate. This is in agreement with the assumption that here the tricarboxylic acid cycle is the main mechanism of acetate oxidation (see also Saz & Krampitz, 1951).

Neurospora crassa. A number of mutant strains of *Neurospora* require succinic acid or related compounds for growth (Lewis, 1948). Succinate can be replaced by fumarate, glutamate, α-ketoglutarate, aspartate, malate and to some extent acetate. Lewis (1948) suggests that the results can be interpreted on the assumption that the tricarboxylic acid cycle operates in *Neurospora*. But it is necessary to make a second assumption, namely that the cycle can be blocked without producing a lethal effect. This means that *Neurospora* must be able to obtain energy from reactions other than the tricarboxylic acid cycle and is in agreement with the idea that in *Neurospora* the cycle is not concerned with energy supply but only with the production of intermediates for synthesis.

Aerobacter agilis. The occurrence of a tricarboxylic acid cycle in *Aerobacter agilis* has been questioned by Karlsson & Barker (1948) on the grounds of 'simultaneous-adaptation' experiments. This organism can oxidize α-ketoglutarate, succinate, fumarate, malate, pyruvate and acetate, and all the enzymes, with the exception of the acetate enzyme, are adaptive. But addition of pyruvate or acetate does not cause adaptation to α-ketoglutarate, and the authors conclude that α-ketoglutarate is, therefore, not an intermediate in the oxidation of acetate. The oxidation of fumarate is likewise slow in cells grown on pyruvate or acetate. Fumarate is therefore also unlikely to be an intermediate.

Multiple functions of enzymic mechanisms

That the chemical constituents of living matter may serve multiple purposes has long been recognized; amino-acids, for instance, are not only constituents of proteins but also precursors of hormones such as adrenaline and thyroxine. The concept that enzymic mechanisms also might serve more than one purpose is supported by general considerations concerning the evolution of biochemical mechanisms. Examples illustrating this are the mechanisms disposing of surplus ammonia in higher organisms, namely the urea synthesis in mammals and amphibia, and uric acid synthesis in birds and nonchelonian reptiles.

Already at primitive levels of evolution, that of unicellular organisms, enzyme systems synthesizing amino-acids and purine bases are present. The former include a system synthesizing arginine, and according to the genetic studies of Srb & Horowitz (1944) and Volcani & Snell (1948), ornithine and citrulline are intermediates in the synthesis of arginine in *Neurospora* and various lactic acid bacteria. The liver of mammals and amphibia utilizes the same type of enzyme systems for the synthesis of urea by coupling it with arginase, which is also present in some micro-organisms. It has been stated that the ornithine cycle occurs in *Neurospora* but, while it is correct to say that the necessary enzyme systems are present, the statement that the cycle takes place makes little sense from the biological viewpoint, since *Neurospora* does not produce urea as an end product of nitrogen metabolism. Ammonia is not toxic to *Neurospora* within wide limits and there is therefore no need for a detoxication of ammonia. The situation is more appropriately described by the statement that *Neurospora* is equipped with the basic enzyme systems required for the synthesis of amino-acids as well as with those needed for the hydrolytic breakdown of cell constituents and that the urea-forming system of the mammalian liver has evolved from these basic mechanisms.

As for the evolution of the uric acid-synthesizing system of birds and reptiles, it is of importance that all animals are capable of synthesizing the purine bases which they require for the synthesis of nucleic acids and nucleotides from small organic molecules and ammonium salts. Many, perhaps all, also possess xanthine oxidase which converts purine bases into urić acid and thereby removes surplus purine bases. Birds form uric acid via the purine bases from the same starting materials—glycine, formate, ammonia, carbon dioxide (Sonne, Buchanan & Delluva, 1948; Buchanan, Sonne & Delluva, 1948)—which other phyla (Heinrich & Wilson, 1950; Shemin & Rittenberg, 1947), and even yeast (Abrams, Hammarsten & Shemin, 1948), use in the synthesis of nucleic acids. By concentrating the same, or very similar enzymes, to a much higher level of activity at special sites, they can employ them for the disposal of all surplus ammonia.

It is the object of these considerations to show that it is not a far-fetched idea to ascribe to the tricarboxylic acid cycle two different functions. Nor are there difficulties in assuming that in some organisms it is the tricarboxylic acid cycle and in others a dicarboxylic acid cycle or still another mechanism which in the course of evolution has developed into the main energy-giving process. Lower organisms which like yeast can grow on acetate as the sole major carbon source are in any case bound to produce a C_4 dicarboxylic acid before the tricarboxylic acid cycle could operate. The mechanism by which a C_4 acid is formed, possibly a dicarboxylic acid cycle, might well have evolved

to become in some species the main energy-producing mechanism. It is also feasible that two mechanisms may occur in the same species side by side, the relative preponderance varying according to environmental conditions. In fact adaptation to new sources of energy may be the adjustment to a second function of a mechanism already present.

SUMMARY

1. The properties of baker's yeast made permeable to di- and tri-carboxylic acids by exposure to dry ice are studied. The effects of cooling on permeability were found to depend on the rate of cooling. Gradual cooling to a temperature of dry ice has no major effect. Sudden transfer from 20–40° to dry ice almost completely abolishes the capacity to oxidize acetate whilst it increases that for oxidizing succinate. Transfer from +2° to dry ice partly preserves the capacity to oxidize acetate, ethanol and glucose. Yeast cells heated for 1 hr. to 50° and then transferred to dry ice have a low blank respiration and do not oxidize acetate, ethanol or glucose but attack succinate and D- and L-lactate.

2. Dry-ice-treated cells are permeable to fumarate, malate and citrate as demonstrated by their high fumarase and aconitase activity. Permeability for succinate is shown by the oxidation of this substance in the cells. The enzymes concerned are all attached to the cells and do not appear in solution.

3. The oxidation of succinate is competitively inhibited by malonate. The inhibition is 50% when the concentration ratio [succinate]/[malonate] is about 60.

4. The oxidation of acetate by dry-ice-treated yeast is not inhibited by malonate under conditions where the oxidation of succinate is inhibited. This result excludes succinate as an intermediate in the oxidation of acetate. Apparent discrepancies between these results and those of Barron *et al.* (1950) and Lynen (1943) are discussed.

5. Simultaneous oxidation experiments with [^{14}C]acetate and non-labelled second substrates exclude succinate, fumarate, malate, α-ketoglutarate, glyoxylate, formate and acetoacetate as intermediates in the oxidation of acetate in dry-ice-treated yeast.

6. Thus, whilst the tricarboxylic acid cycle cannot be the main mechanism of oxidation of acetate in yeast, the component reactions of the cycle can nevertheless occur in yeast cells. Evidence is presented which suggests that the component reactions of the cycle serve to supply intermediates for organic syntheses.

7. A number of observations are discussed which support the view that in many other micro-organisms the reactions of the tricarboxylic acid cycle are primarily concerned with the supply of intermediates, rather than of energy.

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Displacement Chromatography on Synthetic Ion-exchange Resins

8. A SYSTEMATIC METHOD FOR THE SEPARATION OF AMINO-ACIDS

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In this paper it is intended to combine an account of recent work with a survey of the results described in some previous communications in this series (Partridge & Westall, 1949; Partridge, 1949*a*, *b*; Partridge & Brimley, 1951*a*, *b*) in such a way as to present a systematic procedure for the isolation of amino-acids from the hydrolysis products of proteins or from extracts of biological materials. In addition, the results obtained from model experiments with the hydrolysis products of two representative proteins are given; the proteins chosen were egg albumin and a protein fraction extracted from baker's yeast.

The experiments with egg albumin were carried out on what may be regarded as a large laboratory scale, and, in the model experiment described, a batch of 280 g. of the protein was used for hydrolysis. As large quantities of protein were required for these experiments it was convenient to use a commercial grade of egg albumin, but since this product contained considerable quantities of carbohydrate, significant losses of some of the amino-acids were to be expected, due to humin formation during the course of the hydrolysis. It should be emphasized that displacement chromatograms, unlike elution chromatograms, are very flexible as regards the scale on which the experiments are carried out; the amount of material which a column of given dimensions can handle is necessarily much larger with displacement development than it is with elution development, but the most important factor limiting an increase in the scale of an elution chromatogram is the disturbance due to uneven fronts, which is always encountered when broad columns are used. With displacement chromatograms this disturbance may be very largely eliminated by the use of a multiple column, in which the filtration tubes are connected as a series of units of diminishing diameter, and using this device there is no reason to anticipate difficulty in increasing the scale to meet requirements for large quantities of pure bases or amino-acids. In the second model experiment, that with yeast protein, the scale was reduced to approximately one-thirtieth. In this case the weight of the individual amino-acids isolated (from 10 g. of protein) was from 0·1 to 1·0 g., and the scale may be regarded as near the minimum for convenient crystallization and recovery of the less abundant amino-acids.

An important application of any simple method for the isolation of amino-acids from mixtures would lie in the possibility of preparing isotopically labelled amino-acids by a biosynthetic process. It has been suggested (cf. Burris, 1947) that a suitable source of amino-acids labelled with ^{15}N would be the protein from yeast grown with [^{15}N]ammonium salts as the source of nitrogen. The experiment with yeast protein described here was primarily intended as an exploratory step in this direction.

In presenting the systematic procedure it should be emphasized that no single scheme can be the most effective for all mixtures, and various modifications must be made to suit individual requirements. With this in mind, the two examples given show considerable variations in detail, some of which were designed to suit the difference in scale.

APPARATUS AND ION-EXCHANGE MATERIALS

Since columns packed with synthetic resinous ion exchangers may be regenerated and used again many times, it has become the practice to preserve packed filtration tubes as permanent pieces of equipment. In collecting together a set of ready packed columns, there are obvious advantages in a certain degree of standardization as regards height and diameter of filtration tube. It is also useful to supply each packed column with an identification mark and to record data connected with it for use in subsequent experiments. The use of coupled columns of diminishing diameter (Partridge, Westall & Bendall, 1947; Claesson, 1947) has now proved its value for most applications of displacement chromatography (Hagdahl, 1948) and the system has been adopted as a standard one in this laboratory. In addition to its great value in improving the sharpness of the boundaries, the use of standard column sections coupled together also leads to great flexibility. Table 1 shows data for the permanent column sections in use in this laboratory. The standard filtration tubes are numbered I–VII in order of decreasing size, and details are given for three sets of tubes packed respectively with Zeo-Karb 215, sulphonated polystyrene and Dowex 2. It has been shown empirically that the most advantageous column assembly for work with the majority of the amino-acid separations consists of three of the standard filtration tubes coupled in sequence. This scheme is flexible, since a set of three consecutive tubes can always be chosen to accommodate a given weight of mixture without incurring the waste of reagents and time that would be involved in using a column many times too large. Further, if for a particular separation (as, for instance, that of serine and threonine) a column with a very high resolving power is required, accommodation may be made for this by coupling four columns in series. However, in this case a greater quantity of reagents is required, and since the rate of flow must be adjusted to suit the diameter of the smallest column, the time required for the experiment will be much greater than that required for an assembly consisting of three columns.

In order to illustrate the use of the data in Table 1 it may be useful to consider what column assembly would be required for the primary separation of the hydrolysis products of 240 g. of protein using Zeo-Karb 215 as the resin. A rough calculation shows that the mixture would contain about 2000 m-moles of amino-acids and NH_3. The data in Part I of this series (Partridge & Westall, 1949, Fig. 3) shows that 1 g. of Zeo-Karb 215 adsorbs about 2·5 m-moles of amino-acid from isoelectric solution. Thus, provided no free mineral acid were present, 240 g. of protein would require 800 g. of resin for complete adsorption. However, the mixture resulting from the hydrolysis of a protein with HCl always contains a small amount of mineral acid even after careful vacuum evaporation, and as this will diminish the effective capacity of the resin, some accommodation must be made for it. Where amino-acids are adsorbed from isoelectric solution it is usual to leave at least one-third of the column unsaturated in order to secure complete resolution, but experience has shown that, in the primary separation of a protein hydrolysate, it is necessary to increase this proportion to at least one-half. Thus a column assembly containing 1600 g. (dry wt.) of Zeo-Karb 215 would be necessary for the 240 g. batch, and reference to Table 1 (column 4)

Table 1. *Data for a range of laboratory columns*

Filtration tube		Zeo-Karb 215 (capacity for 0·15 N-NH_3, 4·25 mg. equiv./dry g.)					Sulphonated polystyrene (capacity, 5·25 mg. equiv./dry g.)					Dowex 2 (capacity, 3·7 mg. equiv./dry g.)				
(1) No.	(2) Diameter (cm.)	(3) Depth of bed (cm.)	(4) Dry wt. of resin (g.)	(5) Particle size (mesh/in.)	(6) Volume 0·15 N-NH_3 required (ml.)	(7) Flow rate 0·15 N-NH_3 (ml./min.)	(8) Depth of bed (cm.)	(9) Dry wt. of resin (g.)	(10) Particle size (mesh/in.)	(11) Volume 0·075 N-NaOH (ml.)	(12) Flow rate 0·075 N-NaOH (ml./min.)	(13) Depth of bed (cm.)	(14) Dry wt. of resin (g.)	(15) Particle size (mesh/in.)	(16) Volume 0·1 N-HCl required (ml.)	(17) Flow rate 0·1 N-HCl (ml./min.)
I	7·6	61	1100	60–80	30000	—	46	830	60–100	58000	—	—	—	—	—	—
II	5·1	41	335	60–80	9500	40	31	255	60–100	17500	50	—	—	—	—	—
III	3·8	30	138	80–100	3900	20	23	106	60–100	7300	25	23	100	100/200	3700	7·5
IV	2·5	20	41	80–100	1200	10	15	30	60–100	2100	12	15	28	100/200	1000	3·0
V	1·7	13·6	12	80–100	340	4	10	9	60–100	620	6	10	8·3	250/500	310	1·5
VI	1·2	9·6	4·2	80–100	120	1·5	7·2	3·2	80–100	220	2	7·2	3·0	250/500	111	0·75
VII	0·8	6·4	1·3	100–120	37	0·5	4·8	1·0	100–120	70	0·6	4·8	0·9	250/500	33	0·33

shows that the three filtration tubes nos. I–III, coupled in series, would be just sufficient. Column 5 shows the particle size of resin recommended for each filtration tube, and the volume of 0·15 N-NH_3 required as displacement developer may be obtained from the data in column 6; in this case the figure is 43·4 l. The rate of flow of displacement developer chosen for the experiments must be that listed for the smallest column section, i.e. 20 ml./min. (column 7) and thus the time required for application of 43·4 l. of displacement developer will be 36 hr.

was supported by the loosely fitting cork *D*. Closure of the top of the filtration tube was carried out in a similar way.

In order to support the resin bed, and to secure uniformity of flow and thorough mixing, the column was provided with a filtration plate *G*, which fitted loosely inside the filtration tube and rested upon the rubber gasket *E*. The filtration plate was turned from a disk of Perspex and is shown in plan and section in the figure. It was provided with radial and annular grooves in the underside, and was perforated with a large number of evenly spaced holes which

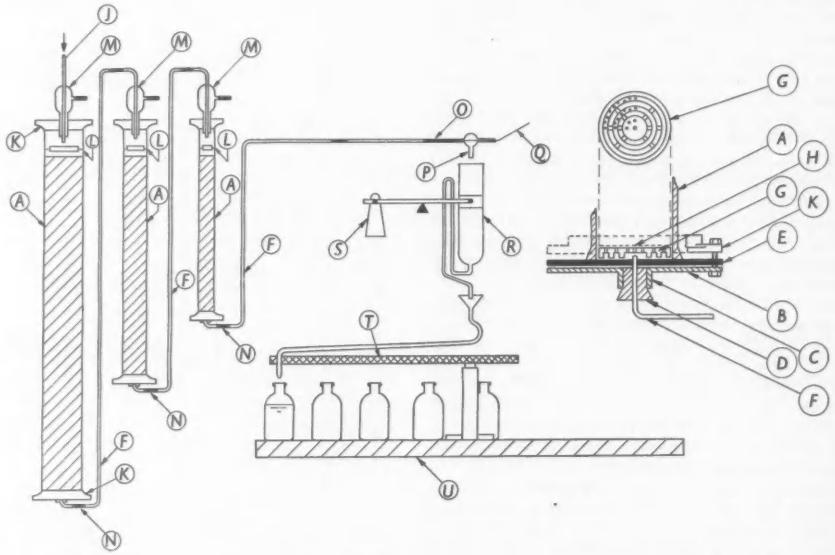


Fig. 1. Construction of the column assembly for the primary fractionation. The key is given in the text.

Construction of the columns. For the smaller column sections (i.e. tubes V–VII, Table 1) a conventional type of construction is suitable. The filtration tubes were made by sealing a glass tap to the end of a glass tube of suitable diameter, and the resin was supported on a plug of glass wool carefully packed into the base. However, with filtration tubes of larger diameter, it was found necessary to make some special provision for ensuring uniformity of flow at the bottom of the column.

The type of construction adopted for larger columns is shown in Fig. 1. The filtration tubes (*AAA*) were made from sections of industrial glass pipeline which were cut to length and provided with standard tapers at both ends by the manufacturers. When used in industrial pipelines the sections are connected together by means of standard metal flanges, and the same flanges (*KK*) were employed for bolting on the endplates in the construction of filtration tubes.

The inset to Fig. 1 shows the endplate *B* which was constructed in metal and provided with a collar *C* to hold the cork *D*. The end of the filtration tube was closed by a thick rubber gasket *E*. This was provided with a small central hole through which was forced the glass outlet tube *F*, thus forming a tight seal. The outlet tube was of narrow bore and passed through the thin sections of the plate left by the grooves. On top of this rested a disk of heavy Fibreglass filter cloth (Fibreglass Ltd., Firhill, Glasgow, N.W.) which prevented the holes in the plate becoming clogged with fine particles of resin. The disposition of the grooves in the plate may be varied to some extent, but should be designed to ensure thorough mixing; however, the provision of a continuous outer ring of full thickness is important since it forms a seal with the rubber gasket and so prevents the passage of fine grains of resin round the edge of the disk.

The arrangement of the apparatus for a large-scale separation experiment is illustrated in Fig. 1. The filtration tubes (*AA*) were arranged side by side and connected by the outflow tubes (*FF*). The solution passed into the apparatus at *J* from a reservoir placed on a shelf arranged at a height about 2 m. above floor level. Each packed column was provided with a float (*LL*) made from a disk of Polythene, which prevented disturbance of the top of the resin by the inflowing solution. In use, the level of solution above the top of the resin was kept as low as was consistent with the proper action of the float. Adjustment of the liquid level was carried out by adjusting the amount of air held at the top of each filtration tube by means of clips placed on the outlet

tubes to the air traps (*MM*). Further clips were supplied at the rubber connections (*NN*) in order to facilitate this operation.

The effluent from the columns passed through a flow regulator (*O*) which consisted of a capillary tube blown to a bulb at one end and provided with an outlet tube (*P*). Regulation of the flow was carried out by varying the depth to which the stainless steel wire (*Q*) was inserted into the capillary. From the flow regulator, the solution passed into the siphon of an automatic fraction collector. This was constructed as described by Brimley & Snow (1949) and consisted of a siphon (*R*) supported on a balance arm carrying a weight (*S*). When the siphon was half full, the balance tipped and operated electrical switches controlling the movement of the arm (*T*). The fraction collector accommodated fifty-six bottles each containing 250 ml. of solution, or a smaller number of 500 ml. bottles. These were arranged in spiral formation on the table (*U*) which was supported about 25 cm. above floor level.

Specification and pre-treatment of the resins

Zeo-Karb 215. The resin, as obtained from the makers (Permutit Co. Ltd., Gunnersbury Avenue, London, W. 4), was dried by spreading it on a sheet of paper in a hot room at 37°. It was then passed through a hammer mill, using a coarse screen, and the product graded by sieving in the dry condition. The sieved material was cleaned by soaking overnight in 5 N-HCl, followed by washing in a beaker with a jet of tap water to remove fine particles.

In packing the column, the filtration tube was first filled with water, and the resin transferred to it in small quantities as a suspension in water. As the particles of resin fell to the bottom of the column water was allowed to flow from the outlet tube below, in order to avoid the disturbance caused by the upward displacement of water by the falling resin. In this way the column was built up as a series of flat laminae, and no attempt was made to consolidate it by tamping or tapping. The column, once made, was not allowed to drain dry, and was always kept with a small depth of water above the resin. Before use, the column was treated two or three times alternately with 2 N-HCl and N-NH_3, and after finally regenerating with 2 N-HCl, was washed with distilled water until the reaction of the effluent reached pH 4.

Sulphonated cross-linked polystyrene. Since the degree of cross linkage of most commercial samples of sulphonated polystyrene is too high for maximum efficiency in the separation of organic bases and amino-acids, the sample of resin used was prepared in the laboratory according to the procedure given by Partridge, Brimley & Pepper (1950). The resin was a cream-coloured substance in the form of microbeads. Its capacity was 5·3 mg. equiv. NaOH/g. of dry H^+ resin. The nominal content of divinylbenzene was 4·5% and the water regain at saturation was 1·8 g. water/g. of dry H^+ resin. The procedure for cleaning the resin and packing the columns was the same as that described for Zeo-Karb 215.

Since completion of the work described here, a sample of lightly cross-linked polystyrene resin, which conformed closely to the above specification, has been received from Permutit Co. Ltd. (London), and this has been used to replace the laboratory-prepared material.

Dowex 2. The resin is a cross-linked polymer containing strongly basic quaternary groups, and was supplied as a cream-coloured material in the form of microbeads (R. W. Greeff and Co. Ltd., 12 Finsbury Circus, London, E.C. 2). In the chloride form the saturation regain was 0·83 g. water/g. dry resin and the capacity for HCl was 3·7 mg. equiv./g. dry resin. Before use the resin was regenerated with carbonate-free 2 N-NaOH until the effluent was free from chloride. (CO_2-free alkali was prepared by adding 10 g. $Ba(OH)_2.8H_2O$/l. of 2 N-NaOH, the solution being filtered immediately before use.) After regeneration the resin was washed with carbonate-free distilled water until the alkalinity of the effluent fell below pH 8. Columns were packed in the same way as described for Zeo-Karb 215, but before use the columns were treated alternately with 0·2 N-HCl and CO_2-free 2 N-NaOH in order to remove impurities and compact the resin bed. Carbonate-free solutions were used at all stages, since it was found that if any carbonate became absorbed on the resin, CO_2 was released by the acid used as displacement developer, and this seriously disturbed the chromatogram.

Although most of the experiments calling for a strongly basic resin have been carried out with Dowex 2, a few of the separations have been successfully repeated with a new product, Deacidite FF (Permutit Co. Ltd.). The capacity of this material was 2·0 mg. equiv. HCl/g. dry Cl^- resin and the water regain at saturation was 1·02 g. water/g. dry Cl^- resin.

EXPERIMENTAL PROCEDURES

In order to effect the complete separation of a complicated mixture of amino-acids and bases by a displacement method it is necessary first to carry out a primary fractionation in which the components are separated as a series of groups; the final separation of the components of the individual groups can then be carried out independently, using for preference a resin with different properties from that used for the group separation. For the primary fractionation the cation-exchange resin Zeo-Karb 215 has many advantages. It is inexpensive and the rate of exchange for large organic cations is very high; large columns can therefore be constructed, and they can be used with high rates of flow. The order of displacement of the amino-acids from Zeo-Karb 215 is also particularly fortunate in that it is markedly different from that given by the strongly basic resins (Partridge & Brimley, 1951*b*) and for this reason most of the amino-acids may be separated by use of the two resins only. However, Zeo-Karb 215 has the disadvantage that it is unsuitable for use at high pH. The resin is of the sulphonated phenol-formaldehyde type and contains phenolic hydroxyl groups. These, at high pH, slowly exchange cations, with the result that the resin shows some degree of irreversible adsorption towards strongly basic substances such as arginine and lysine. Consequently these substances are not quantitatively displaced by stronger bases (Partridge *et al.* 1950).

For this reason, where it is necessary to isolate the strong bases in a mixture, it is desirable to use a cation exchanger which does not contain phenolic hydroxyl groups, and in the present experiments a sulphonated polystyrene resin has been used. However, not all the primary column assembly need consist of polystyrene columns, since during the application of the mixture of amino-acids the strong bases will be concentrated in the top section only; if the top section contains a quantity of sulphonated polystyrene just sufficient to retain all the strongly basic substances, it may afterwards be disconnected from the remainder of the

assembly and developed independently. This procedure has been adopted for the large-scale fractionation described below. For work on a smaller scale it is usually more convenient to carry out the primary fractionation in two stages: in the first stage the whole of the mixture of amino-acids is fractionated on a column of sulphonated polystyrene for the purpose of isolating the basic amino-acids; in the second stage the fraction consisting of the neutral and acidic amino-acids is refractionated on a column of Zeo-Karb 215.

Fractionation of the hydrolysis products of commercial egg albumin

Hydrolysis of the protein. Commercial egg albumin (H_2O, 13·8%; ash, 3·4%; 280 g.) was hydrolysed for 40 hr. under reflux with 3 l. of 5·5 N-HCl. The bulk of the HCl was removed by repeated evaporation with water under reduced pressure and the residue, after addition of water (2 l.), was filtered to remove humin.

Treatment with charcoal. Since the aromatic amino-acids phenylalanine and tyrosine behave rather irregularly on columns of Zeo-Karb 215, it has been found convenient to remove them from the mixture at the outset. This was carried out by treating the slightly acid solution with prepared charcoal. The application of charcoal also served to decolorize the solution.

The charcoal (Activated Charcoal, British Drug Houses Ltd.; 520 g.) was first prepared by shaking for 1 hr. with 5% (v/v) aqueous acetic acid (8 l.) on a mechanical shaker. The mixture was then filtered and the charcoal washed with about 5 l. of distilled water. The protein hydrolysate was made up to 8 l. with distilled water, and the prepared charcoal, which was in the form of a wet filter cake, was transferred to it with stirring. The mixture was then shaken gently for 1 hr. and the charcoal removed by filtration, leaving a colourless filtrate. Finally the charcoal was thoroughly washed with water (8 l.) and the washings added to the filtrate.

Recovery of phenylalanine and tyrosine from the charcoal. The procedure adopted has already been described (Partridge, 1949*a*). The yields of phenylalanine and tyrosine obtained are given in Table 2, together with the analyses of the crystallized products.

Primary fractionation. The column assembly used consisted of three filtration tubes, sizes I–III (Table 1), packed with Zeo-Karb 215 and coupled in series (Fig. 1). Above this was coupled a sulphonated polystyrene column containing 81 g. (dry wt.) of resin (60–100 mesh/in.; filtration tube, size III). The amount of polystyrene resin used for the first column section was calculated to retain the whole of the arginine, lysine and histidine from the protein, assuming these to be 5·7, 6·1 and 2·4% respectively.

The decolorized solution was diluted with distilled water to 40 l.; the pH was then 1·86 (glass electrode). The solution was passed through the column assembly at a rate of

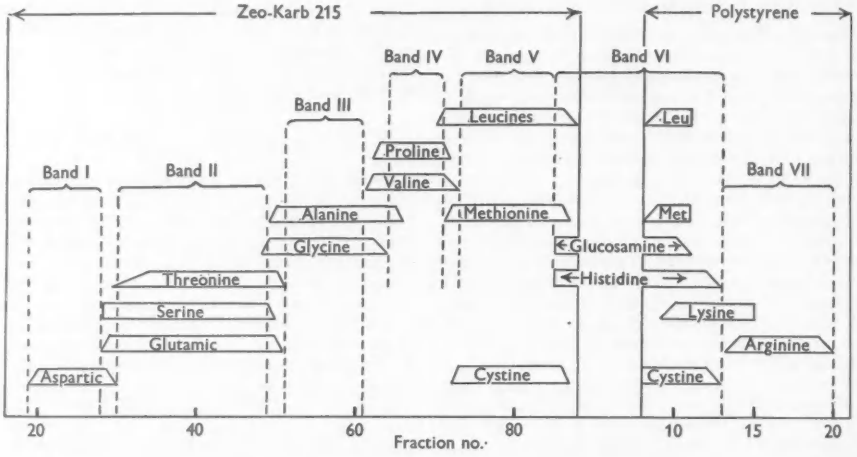


Fig. 2. Primary fractionation of the hydrolysis products of 280 g. of commercial egg albumin. The top section of the column was packed with sulphonated polystyrene and the lower sections with Zeo-Karb 215. The right-hand portion of the figure is the chromatogram resulting from independent displacement of the top section. For experimental details, see text.

40 ml./min., and the effluent solution ran to waste. The column was then washed with a little water and the top section, containing the sulphonated polystyrene resin, was disconnected. The remainder of the column was developed with 0·15 N-NH_3 (44 l.), the solution being passed at a rate of 20 ml./min. The effluent was collected in 250 ml. fractions and 1 drop from each fraction taken for qualitative paper chromatography (Consden, Gordon & Martin, 1944). The result of the qualitative analysis is shown in the left-hand part of Fig. 2.

The sulphonated polystyrene section of the column, which now contained the basic amino-acids, was set up as the top section of a new multiple column, the two lower sections of which were also packed with sulphonated polystyrene resin using filtration tubes IV and V. The amino-acids were then displaced through the two new column sections by applica-

tion of 0·075 N-NaOH as displacement developer; the developer (10 l.) was passed at the rate of 6 ml./min. and the effluent collected in 250 ml. fractions. Analysis of the fractions by paper chromatography gave the result shown in the right-hand part of Fig. 2.

The chromatographic data shown in Fig. 2 were used as the basis for grouping the fractions. The amino-acids fell into a series of mixed bands (numbered I–VII in the figure) which overlapped one another to the extent of two or three fractions. The grouping of the fractions adopted in this experiment is indicated by the dotted lines in the figure; the fractions in each band which lay between the dotted lines were collected together and stored for further fractionation, while those falling in the overlaps between contiguous bands were rejected.

Treatment of the mixed bands. Band VI. This band consisted of the final fractions (126–128) from the Zeo-Karb 215 column and the first fractions (19–23) from the polystyrene column. The combined fractions contained small quantities of leucine, methionine and cystine in addition to the main components, glucosamine, histidine and lysine, but the subsequent fractionation was designed to secure the isolation of the three last-named amino-acids only.

It had been shown previously (Partridge, 1949*a*) that by use of a dilute solution of NaCl as displacement developer the basic amino-acids, including histidine, may be separated sharply from the neutral amino-acids. Under the appropriate conditions, glucosamine and neutral amino-acids are displaced by the front due to Na^+, while histidine, lysine and arginine are retained on the column. The mixed fractions of band VI were accordingly passed through a column of sulphonated polystyrene resin in the hydrogen form (filtration tube III; wt. of resin, 81 g.) and NaCl solution (0·1 N, 5·5 l.) was used as displacement developer. The position of the band comprising glucosamine, leucine and methionine was determined by paper chromatography, and the fractions containing these substances were collected together and set aside for further treatment. The remainder of the fractions were rejected. The glucosamine-rich solution, which contained appreciable quantities of NaCl, was then passed through a column of Zeo-Karb 215 (filtration tubes V–VII) and the organic bases displaced in the usual way by means of 0·15 N-NH_3. The chromatogram showed two bands, the first containing leucine and methionine and the second pure glucosamine. The glucosamine was recovered as the crystalline hydrochloride, yield 1·2 g. The analysis is given in Table 2.

Table 2. *Yields and analyses of amino-acids isolated from commercial egg albumin and from yeast protein*

	Commercial egg albumin (280 g.)				Yeast protein (10 g.)	
	Yield		Nitrogen		Yield	
	(g.)	(% of dry protein)	% found	% calc.	(g.)	(% of dry protein)
Aspartic acid	19·1	8·3	10·6	10·5	0·55	6·4
Glutamic acid	22·0	9·5	9·6	9·5	0·67	7·8
Serine	5·7	2·5	13·4	13·3	0·15	1·7
Threonine	4·9	2·1	11·6	11·8	—	—
Threonine + serine	—	—	—	—	0·43	5·0
Alanine	7·9	3·4	15·7	15·7	0·37	4·3
Glycine	3·3	1·4	18·4	18·7	0·20	2·3
Valine	10·6	4·6	11·9	12·0	0·41	4·8
Proline	2·3	1·0	12·2	12·2	0·26	3·0
Methionine	3·5	1·5	9·5	9·4	—	—
Leucine + isoleucine	18·8	8·1	10·5	10·7	0·23	2·7
Histidine	2·7	1·2	25·6	27·1	0·08	0·9
Lysine	6·8	2·9	12·9*	12·8	0·85	10·0
Arginine	7·8	3·4	31·7	32·2	0·56	6·5
Phenylalanine	5·0	2·2	6·9†	7·0	0·22	2·6
Tyrosine	4·4	1·9	7·8	7·7	0·15	1·7
Glucosamine	1·2	0·5	6·4†	6·5	—	—
Total	126·0	54·2			5·13	59·7

* Analysed as dihydrochloride. † Analysed as hydrochloride.

The basic amino-acids that remained on the sulphonated polystyrene column were recovered by displacement with 0·1 N-NaOH. The fractions collected were free from leucine, methionine and glucosamine but contained a small amount of cystine. Part of the latter crystallized out on storing the solution and the crystals were removed by filtration. A further crop of cystine was then obtained by concentrating the solution under vacuum and allowing it to stand for 2–3 days at 0°. After filtration the solution was decolorized with a little charcoal and mixed with the fractions of band VII for recovery of histidine, lysine and arginine.

Band VII. The solution of basic amino-acids was passed through a column of sulphonated polystyrene resin (filtration tubes III–V) and the column developed with 0·1 N-NaOH. The chromatogram showed three sharply separated bands which contained histidine, lysine and arginine in that order. The fractions forming the narrow overlaps between the bands were rejected, and the pure amino-acids were recovered by crystallization (arginine as the free base, lysine as the dihydrochloride). The yields and analyses are given in Table 2.

Band I. This contained aspartic acid only. The solution was concentrated by vacuum evaporation and set aside to crystallize. The analysis and yield of amino-acid obtained are given in Table 2.

Band II. This contained glutamic acid, serine and threonine. The glutamic acid was separated and recovered by the

method already given (Partridge, 1949*a*). The solution that remained contained serine and threonine only, and these were recovered as a crystalline mixture by addition of warm ethanol to the concentrated solution (yield 21·3 g.). A portion of this mixture (4·9 g.) was taken for the final isolation of L-serine and L-threonine.

Since the isolation of these amino-acids by a displacement method has not been reported previously, the procedure is now given in detail. A survey of the chromatograms obtained with several different resins showed that the serine and threonine bands were in no case completely resolved in experiments at room temperature. Increase in temperature produced rather better resolution in some cases, but after a number of exploratory experiments the conclusion was reached that a separation based on the use of a strongly acidic resin with an aqueous organic solvent offered the best promise. A partial separation on these lines, using Zeo-Karb 215 with 50% acetone-water as the solvent, has already been reported (Partridge, 1949*a*). It was then found that substitution of sulphonated polystyrene resin for Zeo-Karb 215 effected an improvement; however, with short columns the degree of separation of the two bands was still unsatisfactory, and it appeared that even under the best conditions obtainable, the difference in the distribution coefficients of the two amino-acids was insufficient to result in the complete separation of bands of normal length using the standard column assembly.

In order to overcome this difficulty, a column with a higher theoretical-plate equivalent was adopted. This was constructed from routine equipment by coupling a fourth and larger filtration tube above the standard set of three. The new column assembly was operated with the load and rate of flow appropriate to the ordinary assembly consisting of the three lower sections only; thus, while the efficiency of separation was greatly increased, the time required for the separation experiment was increased by a factor of three.

Under these conditions useful separations were obtained, and both serine and threonine were recoverable in a pure condition. However, there was still much overlapping, and in order to ensure a reasonable yield of the two amino-acids it was necessary to carry out the separation in three stages. Using 0·1 N-NH_3 in 50% (v/v) aqueous acetone as displacement developer, a serine-rich and a threonine-rich fraction were first separated on the multiple column, the centre fraction being rejected: the two enriched fractions were then chromatographed independently using the same column and conditions. In this way, 1·3 g. of L-serine and 0·83 g. of L-threonine were obtained from 4·9 g. of the mixture, the yields of the two substances together representing 38% of the weight of mixture taken.

Isolation of L-*serine.* The acetone-water solution from the column experiment was slightly coloured. Acetone was removed by vacuum evaporation, and the solution was decolorized by addition of a little charcoal. After filtration, the solution was concentrated to small bulk and an equal volume of hot ethanol was added. L-Serine crystallized in small prisms, and the product was shown to be free from other amino-acids by paper chromatography. (Found: N, 13·4. Calc. for $C_3H_7O_3N$: 13·3%.) $[\alpha]_D^{21°}$ $-6·45° \pm 0·15°$ in water (*c*, 7·5; *l*, 2 dm.). The yield represented 1·9% of the dry wt. of protein taken.

Isolation of L-*threonine.* After removal of acetone, the solution containing threonine was heated to 70° and treated with a little charcoal. Paper chromatograms confirmed the absence of serine and other amino-acids. The solution was evaporated to the point at which a few crystals appeared and two volumes of hot ethanol were then added. The solution was set aside to crystallize and the product collected by filtration. (Found: N, 11·6. Calc. for $C_4H_9O_3N$: 11·8%.) $[\alpha]_D^{19°}$ $-28·1°$ in water (*c*, 5·1; *l*, 2 dm.). The yield represented 1·6% of the dry wt. of protein taken.

Band III. This consisted of a mixture of glycine and alanine and was uncontaminated with other amino-acids. The mixture was separated by use of a column of Dowex 2 following the procedure given by Partridge & Brimley (1951*b*). The yields and analyses are given in Table 2.

Band IV. The separation and recovery of proline and valine from this band was carried out by fractionating the mixture on a column of Zeo-Karb 215 at 55°, following the method already given (Partridge & Brimley, 1951*a*). The yields and analyses are given in Table 2.

Band V. Two different methods for the isolation of L-methionine and a mixture of the isomeric leucines from this band have already been described. The alternatives are (*a*) the use of a column of Zeo-Karb 215 at a temperature of 55° (Partridge & Brimley, 1951*a*) or (*b*) the use of a column of the strongly basic resin Dowex 2 (Partridge & Brimley, 1951*b*). In this experiment the former method was used since the cation-exchange resin was more readily available in large quantities. No attempt was made to separate the two isomeric leucines, which were obtained as a crystalline mixture free from other amino-acids. The yields and analyses are given Table 2.

Fractionation of the hydrolysis products of yeast protein

Preparation of the yeast protein. Since baker's yeast contains large amounts of carbohydrate it was considered advisable to attempt to extract the protein from the cells, rather than to subject the whole unfractionated material to hydrolysis. However, yeast cells are difficult to disintegrate by the usual procedures, and the results of a few exploratory experiments indicated that partial acid hydrolysis, followed by trichloroacetic acid precipitation of the extract so obtained, offered the simplest procedure for the preparation of the yeast protein in a state of sufficient purity. The procedure proved to be satisfactory for the purpose in mind, but the yield of protein obtained was low.

Wet-pressed baker's yeast was stored for 2–3 weeks at 0° and a portion (500 g.) was heated on a boiling-water bath with 2 l. of 0·2 N-HCl for 90 min. The cell debris was then separated by centrifugation at 3000 rev./min. It was found that when stored yeast was used the supernatant solution was clear, but with fresh yeast an opalescent suspension remained which was only partially cleared by filtration through a layer of Celite (Johns-Manville Co. Ltd., Artillery House, Artillery Row, London, S.W. 1).

The clear solution was mixed with 1 l. of 10% (w/v) trichloroacetic acid solution and the precipitated protein collected by centrifugation. This was washed several times with acetone and finally with dry ether. The residual ether was removed under reduced pressure at 60° and the product dried *in vacuo*. In this way the crude protein (10 g.) was obtained as a fine white powder. (Found: N, 14·1; moisture, 12·6; ash, 0·9%.) The product gave a positive Molisch test.

Hydrolysis of the protein. The crude yeast protein (10 g.) was hydrolysed with 5·5 N-HCl following the procedure

given for egg albumin. A two-dimensional chromatogram showed the presence of aspartic acid, glutamic acid, serine, threonine, proline, valine, glycine, alanine, leucine, histidine, lysine, arginine, tyrosine and phenylalanine, but cystine and methionine were present as traces only. The hydrolysis product was treated with prepared charcoal (17·2 g.) following the procedure described above. A paper chromatogram carried out with the colourless filtrate after charcoal treatment showed the absence of tyrosine and phenylalanine.

Bands VI–VIII. At this stage, bands VI–VIII were worked up for histidine, lysine and arginine respectively. The lysine and arginine bands required no further fractionation and the crystalline amino-acids were recovered by the methods already given. The histidine band contained, in addition, leucine, lysine and a small amount of cystine. The solution was passed through a column of Dowex 2 (filtration tubes V and VI) and the column was displaced with 0·075 N-HCl collecting approx. 4 ml. fractions. The first 10 fractions (fractions 40–49) contained lysine only, and these were

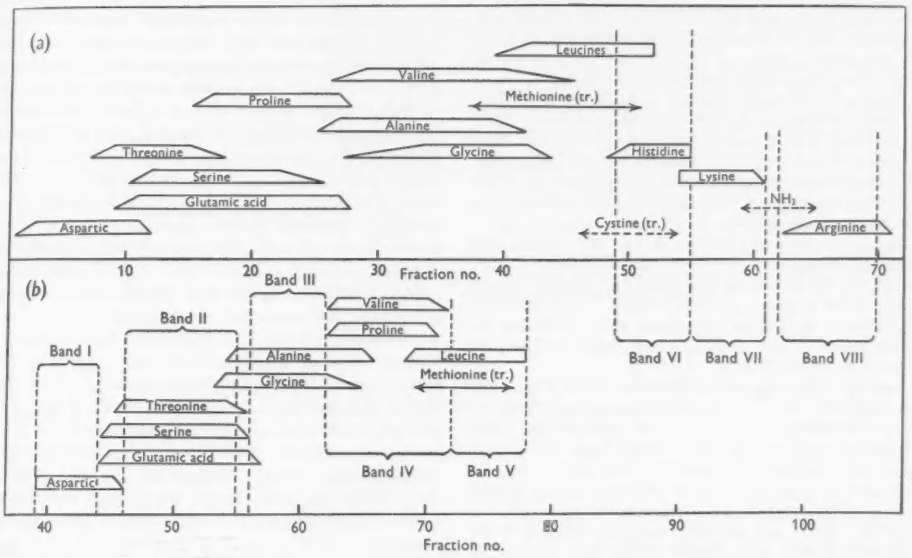


Fig. 3. Primary fractionation of the hydrolysis products of 10 g. of yeast protein. (*a*) Chromatogram prepared by displacing the mixture from a column of sulphonated polysytrene with 0·075 N-NaOH. (*b*) Fractions 1–49 from chromatogram (*a*) adsorbed on a column of Zeo-Karb 215 and displaced with 0·15 N-NH_3. For experimental details see text. tr. = trace.

Recovery of phenylalanine and tyrosine. Crystalline phenylalanine and tyrosine were recovered from the charcoal by elution with phenol-acetic acid mixture following the method given above; the yields are given in Table 2. A paper chromatogram carried out on the phenol-acetic acid eluate showed no amino-acids other than phenylalanine and tyrosine.

Primary fractionation: first stage. In this experiment the primary fractionation was carried out in two stages. In the first stage the charcoal-treated filtrate (1·5 l.) was passed through a column of sulphonated polystyrene resin (filtration tubes IV–VI) and the column developed with 0·075 N-NaOH. The fractions (15 ml.) were collected by use of a clockwork-driven device (Snow, 1951) which changed the receivers at fixed intervals of time (20 min.). The fractions were analysed by paper chromatography with the result shown in Fig. 3 (*a*). The chromatogram shows complete separation of arginine and lysine, and it is noteworthy that the ammonia band falls between the two amino-acids, thus improving the resolution by acting as a 'carrier' (cf. Tiselius, 1943; Tiselius & Hagdahl, 1950).

added to the fractions of band VII for lysine recovery. Fractions 50–61 contained lysine, leucine and a small amount of histidine, and were rejected. The histidine appeared in pure solution in fractions 62–65 and was followed by a further four fractions containing cystine and HCl. Crystalline histidine was recovered from fractions 62–65; the yields of the three basic amino-acids are given in Table 2.

Isolation of serine. Fig. 3 (*a*) shows considerable separation of serine from threonine, and advantage was taken of this to secure the isolation of part of the serine present in the mixture. Fractions 19–25 (Fig. 3 (*a*)) were passed through a column of Dowex 2 (filtration tubes V and VI) and displaced with 0·075 N-HCl. A clear separation of proline, serine and glutamic acid was obtained, the serine appearing in the centre fraction. The serine was crystallized from the pure solution and the mother liquors were combined with the fractions containing proline and glutamic acid for re-addition to the main stock. The yield of serine was 0·15 g.

Primary fractionation: second stage. Fractions 2–49 (Fig. 3 (*a*)) were bulked together and passed through a

column of Zeo-Karb 215 (filtration tubes IV–VI). The displacement developer was 0·15 N-NH_3 and the chromatogram obtained (15 ml. fractions) was as shown in Fig. 3 (*b*). The chromatogram showed five sharp bands with very little dislocation of the individual amino-acids within the bands. The fractions were collected together as indicated by the dotted lines in the figure, and the overlapping portions of the bands were rejected. The treatment of the individual bands is summarized below, and the yields of the crystalline amino-acids isolated from each band are given in Table 2.

Band I. The aspartic acid contained in this band was free from other amino-acids and was isolated by crystallization.

Band II. The mixture of serine, threonine and glutamic acid was passed through a column of Dowex 2 (filtration tubes V and VI) and displaced with 0·075 N-HCl. The amino-acids separated sharply into three bands. The first contained the small amount of glycine and alanine present in the mixture and was rejected. The second band contained serine and threonine in pure solution, and the two amino-acids were recovered together as a crystalline mixture. The final band consisted of pure glutamic acid which was recovered by crystallization.

Band III. The mixture of glycine and alanine was separated on a column of Dowex 2 (filtration tubes V and VI) using 0·1 N-HCl as displacement developer. Separation was sharp and the alanine band appeared first. The glycine band was followed by a trace of glutamic acid, which was rejected. The two amino-acids were recovered from their pure solutions by crystallization.

Band IV. This consisted of fractions 63–73 (Fig. 3 (*b*)). The separation of the valine-proline band was insufficient to allow the recovery of the two amino-acids in good yield. Accordingly, the mixed fractions from the band were treated again with Zeo-Karb 215, using a column assembly of just sufficient size to accommodate the mixture (filtration tubes V–VII). Ammonia solution (0·15 N) was used as displacement developer, and the chromatogram obtained (8 ml. fractions) showed a sharp separation into three bands. The first band contained glycine and alanine (fractions 3–5) and the pure solution containing these amino-acids was returned to the stock from Band III. Fractions 6–9 represented the overlapping portion of the first and second bands, and were rejected. Fractions 10–20 contained the mixture of proline and valine free from other amino-acids, while fractions 21–23 contained leucine in addition to valine and were rejected.

The mixture of valine and proline was then passed through a column of Dowex 2 (filtration tubes V and VI) and displaced with 0·1 N-HCl. A sharp separation was obtained and valine and proline were recovered by crystallization.

Band V. This contained leucine, isoleucine and a small amount of methionine. The mixture was passed through a column of Dowex 2 (filtration tubes V and VI) and displaced with 0·1 N-HCl. The chromatogram showed a long band consisting of the isomeric leucines followed by a short band containing methionine and a trace of phenylalanine. The amount of methionine was too small for convenient recovery, and the solution was rejected. A crystalline mixture of the two isomeric leucines was recovered from the bulked fractions comprising the first band.

DISCUSSION

The two examples given in the experimental part of this paper were intended to illustrate the application of a systematic procedure suitable for the isolation of individual amino-acids and bases from the complicated mixtures occurring in protein hydrolysates, tissue extracts or secretory fluids. The procedure adopted in the two examples differed in detail, and it is not possible or desirable to lay down a completely finalized routine that could be applied inflexibly to all problems: such a routine would necessarily prevent full advantage being taken of the favourable circumstances of individual problems. Nevertheless, the second example represents a less empirical approach, and has so far proved to be a convenient procedure for all but very large-scale work. In this procedure three resins were used, and the separation was carried out in three main stages. (1) The amino-acid hydrochlorides were adsorbed on a column of sulphonated polystyrene resin and displaced with a solution of sodium hydroxide. This step was included for the specific purpose of isolating arginine, lysine and histidine; if these three amino-acids or other strong bases are absent, or their isolation is not desired, the stage may be omitted. (2) The fractions of the eluate from stage (1) containing the acidic and neutral amino-acids were combined, and the solution passed through a column of Zeo-Karb 215. The column was then displaced with dilute ammonia solution. This completed the 'primary separation' and resulted in a series of mixed bands which were very suitable in composition for the final separation by means of a strongly basic resin. (3) Each of the mixed bands from stage (2) was passed through a column of Dowex 2 and displaced with dilute hydrochloric acid.

The application of this procedure resulted in the isolation of all the amino-acids of the protein hydrolysate except two pairs which were not readily resolved. These were leucine-isoleucine and threonine-serine. The separation of the isomeric leucines was not attempted, but in view of the difficulty of obtaining pure L-serine and L-threonine by conventional methods, the separation of these two substances was studied in some detail. After a number of trials, the most convenient procedure found consisted in displacing the mixture from a column of sulphonated polystyrene resin, using sodium hydroxide dissolved in 50 % aqueous acetone as the displacement developer.

No example of the separation of amino-acids from biological extracts has been given in this paper, but an excellent procedure for the primary separation has been described by Westall (1950). Biological extracts usually contain materials, in part of high molecular weight, such as proteins, polysaccharides, tannins and anthocyanins, which must first be removed. The procedure adopted for deproteinization should not result in the addition of large amounts of inorganic salts to the extracts, and

for small-scale work dialysis, or the addition of ethanol to a concentration of about 80% (v/v), is suitable. Deproteinization may be followed by treatment with charcoal to remove phenolic substances, colouring matter and the aromatic amino-acids. On a large scale, the use of ethanol for deproteinization is inconvenient; the addition of a predetermined amount of basic lead acetate, following the procedure given by Westall (1950) is preferable.

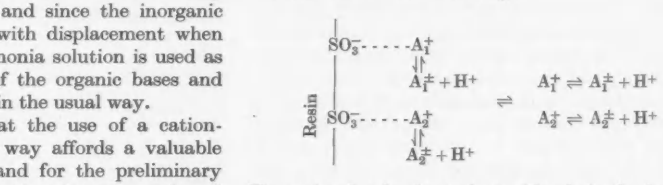
Once the components of high molecular weight have been removed from the mixture, the treatment applied may be substantially the same as that adopted for protein hydrolysates. The solution will usually contain inorganic salts, organic acids, sugars and other neutral substances in addition to amino-acids and bases; but on passing the mixture through a column of a strongly acidic resin in its hydrogen form, the inorganic cations remain at the top of the column while the organic and inorganic anions are not adsorbed and pass through as free acids together with the neutral substances. After washing with water, the column contains only cations and ampholytes, and since the inorganic cations do not interfere with displacement when sodium hydroxide or ammonia solution is used as developer, the isolation of the organic bases and amino-acids may proceed in the usual way.

It should be noted that the use of a cation-exchange column in this way affords a valuable procedure for desalting, and for the preliminary separation of organic extractives into groups based on their ionic charge (cf. Platt & Glock, 1943; Partridge, 1948). The first fraction of the effluent from the cation-exchange column contains the organic and inorganic acids and the sugars, and these groups may again be separated by passage through an anion-exchange column. It is often convenient to carry out a qualitative analysis on each of the groups separately by means of paper chromatography, and to follow this by the application of further column procedures for the isolation or quantitative determination of individual components.

Another important application of the procedure would lie in the preparation of amino-acids and bases labelled with ^{15}N, ^{13}C or ^{14}C. Proteins so labelled may readily be prepared biosynthetically by growing micro-organisms on synthetic media containing the required labels in inorganic form. The experiment with yeast protein was carried out as an exploratory step in this direction, and the example given demonstrates the practicability of isolating the amino-acids in good yield from amounts of crude protein of the order of 10 g. Yeast is not an ideal organism for work of this kind, since it contains a large amount of carbohydrate which is difficult to remove from the protein; it may be more convenient in future work to choose an organism containing a lower amount of carbohydrate, and to subject the whole organism to hydrolysis without prior isolation of a protein fraction. This procedure has been adopted with success in recent work carried out in this laboratory in connexion with the preparation of amino-acids labelled with ^{14}C.

The relationship between the ionic charge of a solute and its position in the order of displacement has been discussed by Davies (1949), and further discussion has since appeared in papers in this series. Extension of the experimental work to cover a larger range of solutes and several different resins has provided confirmatory evidence for the mechanism proposed by Davies and permitted its extension to the behaviour of anion- and cation-exchange resins with mixtures containing multivalent ampholytes.

Conditions during the separation of a mixture of monocarboxylic monoamino-acids using a strongly acidic cation-exchange resin may be illustrated by a scheme such as the following:



The resin adsorbs the amino-acid only in the form of the cation A^+ and this form is in reversible equilibrium with hydrogen ions and uncharged molecules in the surface layer. The system at the surface of the resin is again in equilibrium with the solution remote from it, but since the resin surface is charged, the concentration of hydrogen ions at the interface will be different from that in the ambient solution.

In the scheme given above, the displacement of the weaker base A_2 will depend primarily upon the partial suppression of the cationic form of A_2 by the presence of the stronger base A_1. However, differences in the affinity of the resin for the cations A_1^+ and A_2^+ will also be important and must be taken into account. This 'adsorption affinity' may be regarded as the sum of two factors: the electrostatic attraction of the two charged centres and the contribution of van der Waals forces. The electrostatic term contributes very largely to the total adsorption energy and may be modified by the valency of the ion, by hydration and by factors affecting the ion diameter. The van der Waals term, on the other hand, will increase with increasing molecular weight and will be very sensitive to structural factors such as the presence or absence of an aromatic ring.

A study of the order of displacement of a range of solutes confirms that the main factor in determining

Table 3. *Dissociation equilibria governing adsorption on cation- and anion-exchangers, given for a number of ion types*

	Cation exchange		Anion exchange	
Monocarboxylic monoamino	$A^+ \rightleftharpoons A^{+,-} + H^+$	pK_1	$A^{+,-} \rightleftharpoons A^- + H^+$	pK_2
Dicarboxylic monoamino	$A^+ \rightleftharpoons A^{+,-} + H^+$	pK_1	$A^{+,-} \rightleftharpoons A^{+,2-} + H^+$	pK_2
Monocarboxylic diamino	$A^{2+,-} \rightleftharpoons A^{+,-} + H^+$	pK_2	$A^{+,-} \rightleftharpoons A^- + H^+$	pK_2
Dicarboxylic diamino	$A^{2+,-} \rightleftharpoons A^{2+,2-} + H^+$	pK_2	$A^{2+,2-} \rightleftharpoons A^{+,2-} + H^+$	pK_2
Univalent base or acid	$BH^+ \rightleftharpoons B + H^+$	pK	$A \rightleftharpoons A^- + H^+$	pK
Bivalent base or acid	$BH^+ \rightleftharpoons B + H^+$	pK_2	$A \rightleftharpoons A^- + H^+$	pK_1

the order is the basic or acidic strengths of the solutes, and it has become the practice to regard any departures from this order based on pK values as 'anomalous'. An estimate of the expected position of a solute in the order of displacement is of considerable value in experimental work, and this may be obtained provided the dissociation constants of the solute are known. Table 3 lists the relevant equilibria and ionization constants governing the adsorption of a number of ion types by a strongly acidic and a strongly basic resin. The dissociation exponents given are the negative logarithms of the acid dissociation constant for each reaction.

In Table 4 the experimentally determined order of displacement of a range of ionic solutes from a strongly acidic cation-exchange resin and a strongly basic anion-exchange resin is given. The relevant pK values, taken from Cohn & Edsall (1943), are included for comparison. It will be observed that the order of displacement is substantially the order of pK values, but in several cases (those marked with an asterisk) a solute is more strongly retained than would be expected from its dissociation constant. The aromatic amino-acids show marked anomalous adsorption, and proline and methionine are also retarded on both types of resin. In Table 4 amino-acids which form mixed bands in the routine procedure are bracketed together, and it will be observed that, with the cation-exchange resin, the resolution of six discrete bands may be observed over the pK range 1·88–2·36. Since successful resolution may be obtained over the full range to about pK 10·6 it is clear that the displacement method is capable of resolving very complicated mixtures of bases, and that the separation of bases in the range pK 3–9 could proceed without interference from neutral or acidic amino-acids present in the mixture. A similar situation holds for the strongly basic resin Dowex 2, where separations in the range pK 8–3 have received little investigation.

Recently, Stein & Moore (1950) and Moore & Stein (1951) have described a very elegant procedure for the chromatographic fractionation of mixtures of amino-acids by elution analysis, using columns of a commercial sulphonated polystyrene (Dowex 50). This procedure is more suitable for

Table 4. *The sequence of displacement of a range of solutes from two resins at room temperature*

(The solutes are arranged in sequence of displacement from the resins. Relevant pK values are from Cohn & Edsall (1943). Values not falling in the sequence are marked with asterisks (see text).)

Sulphonated cross-linked polystyrene (4·5% divinylbenzene)		
Aspartic acid	pK_1	1·88
Hydroxyproline	pK_1	1·92
Threonine	—	—
Serine	pK_1	2·21
Glutamic acid	pK_1	2·19
Proline	pK_1	1·99*
Glycine	pK_1	2·34
Alanine	pK_1	2·34
Valine	pK_1	2·32
Methionine	pK_1	2·28*
Leucine	pK_1	2·36
Cystine	pK_2	2·26*
Creatine	pK_1	approx. 3·0
Phenylalanine	pK_1	1·83*
β-Alanine	pK_1	3·60
Trimethylamine oxide	pK	approx. 4·5
Creatinine	pK	approx. 4·8
Histidine	pK_2	6·0
Methylhistidine	pK_2	6·48
Carnosine	pK_2	6·83
Anserine	pK_2	7·04
Hydroxylysine	—	—
Lysine	pK_2	8·95
Ammonia	pK	9·27
Arginine	pK_2	9·04
Methylamine	pK	10·64
Dowex 2		
Lysine	pK_3	10·53
Proline	pK_2	10·60*
β-Alanine	pK_2	10·19
Alanine	pK_2	9·69
Valine	pK_2	9·62
Leucine	pK_2	9·60
Glycine	pK_2	9·60
Carnosine	pK_2	9·51
Threonine	—	—
Serine	pK_2	9·15
Histidine	pK_2	9·17*
Methionine	pK_2	9·21*
Methylhistidine	pK_2	8·85
Cystine	pK_3	7·85
Tyrosine	pK_2	9·11*
Acetic acid	pK	4·75
Glutamic acid	pK_2	4·25
Aspartic acid	pK_2	3·65
Hydrochloric acid	—	—

quantitative analysis than the displacement method, and it may also be used for isolation work on a small scale (cf. Ehrensvärd, Reio, Saluste & Stjernholm, 1951).

The formation of elution peaks depends upon the presence in the developing solution of cations capable of competing with the cationic form of the amino-acids. The competing cations are hydrogen ions in the case of development with acids, or sodium ions in the case of development with sodium buffers; a peak may thus be caused to travel more rapidly by increasing the ionic strength of the developing solution in either case. As would be expected from the theory of Davies (1949), acceleration of a peak may also be induced by increasing the pH of the developer, thus suppressing the cationic form of the amino-acid. When the solvent used for elution development is a buffer solution, the pH of the ambient fluid is predetermined, and in the procedure worked out by Moore & Stein the developing solvents consist of a range of buffers from pH 3·4 in the early stages to pH 11·0 in the final stage. In the displacement chromatogram, on the other hand, the pH of the ambient fluid is controlled only by the presence of the amino-acids themselves; here likewise it is found that during the fractionation of a range of amino-acids the pH of the first fractions (containing aspartic acid) is about 3·6; the pH then rises by a series of steps to about pH 10 in the region of lysine. It is not surprising therefore that the sequence of the elution peaks in the method of Moore & Stein is very similar to that obtained in displacement chromatography, and that both sequences suffer the same alterations and reversals when experimental conditions such as temperature are changed. This situation is a convenient one since it may prove that experience gained with one method may be applied to the other.

It is encouraging to observe that a very large range of ampholytes and bases may be separated by the use of synthetic resins of very simple structure. In the preparation of these resins no deliberate attempt has been made to introduce chemical configurations which are likely to confer specific adsorptive effects, and the fact that the application of both the elution and the displacement procedures has resulted in the separation of closely related homologues suggests that the methods are capable of far wider application. The majority of substances of physiological importance are water-soluble and ionic in character, and there appears to be every reason for the expectation that many other groups of substances of biological origin could be fractionated chromatographically with only minor modifications of the same procedures.

SUMMARY

1. A systematic procedure is described for the fractionation and isolation of amino-acids and bases using displacement chromatograms carried out with synthetic ion-exchange resins.

2. The procedure is illustrated by two model experiments. In the first example 16 amino-acids were isolated from 280 g. of commercial egg albumin with an overall recovery of 54 % of the dry weight of protein taken. In the second example 10 g. of a protein derived from yeast were hydrolysed and fractionated. Thirteen crystalline amino-acids were recovered, the total weight representing 60 % of the dry weight of the protein.

3. A new procedure for the isolation of threonine and serine is described.

4. The relationship between the dissociation constants of a solute and its position in the sequence of displacement is discussed.

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Degradation of Protein in the Rumen of the Sheep

1. SOME VOLATILE FATTY ACIDS, INCLUDING BRANCHED-CHAIN ISOMERS, FOUND *IN VIVO*

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Work has been in progress here for some years on the use made by the sheep of different kinds of protein under different conditions (to be published; for preliminary communications on certain aspects see Cuthbertson & Chalmers, 1950; Chalmers & Synge, 1950). The results obtained have laid stress on the degradation of protein nitrogen to ammonia in the rumen. The possible importance of this was minimized in a review article by Schieblich (1929) and seems to have been first explicitly recognized by Pearson & Smith (1943) on the basis of *in vitro* incubation of protein with rumen contents. Detailed physiological data, however, were first obtained by McDonald (1948*a*, *b*) who measured the concentration of ammonia in the sheep's rumen at different times after feeding various proteins, and also showed that appreciable quantities of this ammonia may be absorbed into the blood draining the rumen; the ammonia would then presumably to a large extent be converted to urea in the liver and so be excreted. McNaught & Smith (1947) and Elsden & Phillipson (1948) have reviewed nitrogen metabolism in the rumen.

The studies made at this Institute have in fact shown that nitrogen retention and growth in sheep on various diets can be correlated inversely with the extent of ammonia production in the rumen. In parallel with this work it seemed desirable to try to find out what other products of protein breakdown are formed besides ammonia. The present paper describes some relevant *in vivo* phenomena observed by chemical analysis of samples of rumen liquor obtained from sheep in the course of experiments on protein utilization by Dr Margaret Chalmers and Dr R. L. M. Synge. The subsequent paper describes *in vitro* experiments with the mixed micro-organisms of such rumen liquors, which throw light on some of the chemical reactions undergone by protein during its degradation in the rumen. This work formed the subject of a Thesis submitted to Aberdeen University for the Ph.D. degree in 1951 and some of it was included in a preliminary communication (el-Shazly & Synge, 1950).

It seemed probable, from information available on bacterial deamination of amino-acids (cf. el-Shazly, 1952), that volatile fatty acids (VFA's) might be formed at the same time as ammonia. The higher members of the normal series present in the rumen (C_4 and higher) might be expected to arise to a significant extent from amino-acids as well as from carbohydrates; members of the branched-chain series, arising from the amino-acids valine, leucine and isoleucine, which usually contribute about 20 % of the nitrogen of proteins, would not be expected to arise at all from carbohydrates. When this work was begun in 1948, none of the micro-methods available for analysing VFA's gave a good separation of branched- and straight-chain isomers. Some of the C_4 and (C_4 and higher) fractions isolated by the procedure of Elsden (1946) were therefore subsequently examined by the oxidation method of Klinc (1934, 1938). The results so obtained, though not inconsistent with the more recent findings, are not given here since the gas-liquid partition chromatogram of James & Martin (1951, 1952) became available later; this permitted unequivocal resolution of branched- and straight-chain isomers and of C_5 from C_6 and higher homologues, as well as being both rapid and convenient in use.

METHODS

VFA's used for control purposes were A.R. or 'laboratory reagent' quality (British Drug Houses Ltd.). They had the calculated equiv. wt. on titration with alkali and were homogeneous by the chromatographic methods mentioned below.

Rumen liquor. Samples were taken by Dr Margaret I. Chalmers, Dr A. T. Phillipson or Dr R. L. M. Synge from Cheviot ewes having permanent rumen fistulae, by aspiration through a glass or Polythene tube. The samples were strained through muslin and cooled quickly to 0°. The fistula operations had been done by Dr A. T. Phillipson.

Determination of ammonia in rumen liquor. 5 ml. of strained liquor and 5 ml. 0·2 N-HCl were mixed, diluted to 50 ml. with water and filtered (cf. McDonald, 1948*a*). NH_3 was determined in the filtrate according to Conway & O'Malley (1942), adding 0·5–1·0 ml. of filtrate to 1 ml. saturated aqueous K_2CO_3 in the outer chamber of the diffusion cell.

Preparation of rumen liquor for analysis of carboxylic acids. In view of the special interest in the C_4 and higher VFA's, and other acids which might be retained by particles of the rumen liquor on their removal, tests were made comparing

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the recovery of hexanoic acid added to rumen liquor: (*a*) after dialysis at rumen pH where diffusion would be as the anion; (*b*) on precipitation of particles, protein etc. by $MgSO_4$–H_2SO_4 according to the general procedure of McAnally (1944).

Portions (28–30 mg.) of hexanoic acid (as Na salt) were added to 19–25 ml. portions of rumen liquor. Control portions of rumen liquor without added hexanoate were treated in what follows in the same way. (*a*) The samples were dialysed in cellophan sacs at 0° for 36 hr. with intermittent stirring against sufficient distilled water to give a total vol. of 100 ml. (*b*) Water (25 ml.) was added to the samples and then 50 ml. of 2·5% (v/v) H_2SO_4 in sat. aqueous $MgSO_4$. After keeping overnight at 0° the mixture was filtered. Known volumes of (*a*) diffusate or (*b*) filtrate were analysed for (C_3 and higher) VFA's by Elsden's method after distillation or extraction (see below). The differences found, recalculated to the original samples, were taken to represent the recovery of added hexanoic acid, which was (*a*) 83–88%; (*b*) 83%. It might be expected that VFA's lower than C_6 would show higher recoveries. Recovery experiments with added *n*-butyric acid by method (*b*) gave, in fact, values in the range 82–102%. Accordingly rumen-liquor samples were prepared for analysis throughout the work according to method (*b*). The recoveries of the acids originally present may not have been as good as those of the added acids, but the results should at least have significance for comparative purposes. For obtaining VFA's the filtrates were distilled with steam in all-glass apparatus and successive portions of distillate were titrated with NaOH until no more acid distilled. Some determinations of total acids extractable by ether from the filtrate were made using a continuous liquid-liquid extractor. As well as giving good recoveries of VFA's, control experiments showed good recoveries of added succinic acid; it is apparent from inspection of the data collected by Washburn (1928) that several other well known physiologically important polycarboxylic acids, hydroxy acids, keto acids etc. would, if present, have been recovered in fair yield. However, on removal of VFA's by steam distillation from these ether extracts of rumen-liquor filtrates it was found that the non-volatile ether-extractable acids never exceeded 2·5% (equiv. basis) of the total VFA present.

In view of their favourable partition coefficients extraction by ether was sometimes used instead of steam distillation for convenience in isolating the (C_4 and higher) VFA's when it was wished to determine only these components. The ether extracts were titrated with aqueous NaOH, the ether was removed by evaporation and the residues were analysed in the same way as the steam distillates. McClymont (1951) has used a similar procedure.

Determination of VFA's by liquid-gel partition chromatography. The Na salts were prepared for chromatography and analyses were carried out as described by Elsden (1946). VFA's of group (C_5 and higher) were sometimes not easily separated from the immediately following C_4 band, but no uncertainty attaches to the totals for (C_4 and higher) VFA's.

Determination of VFA's by gas-liquid partition chromatography. This was done as described by James & Martin (1952) using a 4 ft. column of internal diameter 4 mm.; 0·1 parts by weight of stearic acid were added to the silicone and the column was operated at 137° with manual control of the recording microburette. The pressure of N_2 was adjusted to give 15 ml./min. of effluent gas. Solutions of mixed Na salts obtained as described above were concentrated to approx. 2N. Portions of this solution (30 µl.) were then pipetted on to a plug of pyrophosphoric acid-kieselguhr mixture in a short tube of the same bore as the gas-liquid chromatogram. The proportion of kieselguhr was adjusted so that the mixture stayed powdery on adding the liquid. More of the same mixture was then packed behind the sample, and the short tube was connected (rubber sleeve over close-fitting glass ends) to the inlet end of the gas-liquid chromatogram. By heating the plug tube and joint to 100° and applying vacuum to the outlet end of the gas-liquid chromatogram (which was maintained at room temperature) the free VFA's were distilled into the chromatogram tube as an anhydrous mixture. The chromatogram tube was then placed in its vapour jacket and the analysis was conducted without delay. This procedure was used by James & Martin (private communication) at one stage in their development of the method, but is now superseded (James & Martin, 1952). Aggregate recoveries of control mixtures of VFA's via the Na salts were mostly in the range 90–110%. Errors were presumably in measurement of samples on to the pyrophosphoric acid, by absorption of VFA's in the rubber tube, carry-over of traces of water, etc. Errors when free VFA's were applied directly to the chromatogram were considerably less. In both cases the errors in the relative proportions of the VFA's, the main interest of the present study, were smaller. α-Methylbutyric acid ('active valeric acid') was presumably measured with *iso*valeric acid, since the 4 ft. column used was not sufficient for its resolution. The commercial '*iso*valeric acid' used for control purposes was presumably also a mixture of these acids (see James & Martin, 1952).

RESULTS

Feeding of sheep

The daily dietary regime for the various experiments is given in Table 1, and the protein and dry-matter contents of the feeding stuffs are given in Table 2. Unless otherwise stated, the animals had been on the same diet for at least a fortnight before the day of the experiment. Feeds were nearly always consumed within half an hour of being given.

Time course of evolution of ammonia and higher volatile fatty acids

In Exps. 1–6 the content of ammonia and of C_4 and (C_5 and higher) VFA's (determined by the procedure of Elsden) was determined in rumen-liquor samples taken at different times, expressed as mg. equiv./100 ml. rumen liquor and plotted against time (Figs. 1–3). The results show a marked parallelism in the evolution of ammonia and of the VFA's, especially (C_5 and higher).

Recognition of presence of branched-chain C_5 volatile fatty acids

Figs. 4 and 5 show analyses, by the method of James & Martin, of typical rumen-liquor VFA samples before and after the addition, respectively,

Table 1. *Dietary regime of fistulated Cheviot ewes*

(Weights of food are air dry, as fed. Analytical and other data on the foods are given in Table 2.)

		Time of feeding						
		07.00 hr.		11.00 hr.		16.00 hr.		
Exp. no.	Sheep no.	Food	Wt. (g.)	Food	Wt. (g.)	Food	Wt. (g.)	Date
1	716	Hay I	240	Hay I	240	Hay I	240	5. viii. 49
2	717	Concentrate I Casein	150 50	Hay I	200	Hay I	200	5. viii. 49
3	718	Concentrate I	150	Hay I	200	Hay I	200	21. iii. 49
4	716	Concentrate I Casein	150 50	Hay I	200	Hay I	200	21. iii. 49
5	716	Fresh grass	800	Fresh grass	800	Fresh grass	800	24. v. 50
6	718	Fresh grass	800	Fresh grass	800	Fresh grass	800	24. v. 50
7	718	Concentrate II Casein	600 50	Hay II	300	Hay II	300	5. xii. 50
8	718	Concentrate II Casein	600 50	Hay II	300	Hay II	300	12. xii. 50
9	722	Concentrate II Casein	400 100	Hay II	300	Hay II	300	5. xii. 50
10*	719	Concentrate II Casein	400 100	Hay II	300	Hay II	300	12. xii. 50
11, 12	719	Frozen grass	1600	Frozen grass	1600	Frozen grass	1600	19. x. 50 and 31. x. 50
13, 14	722	Dried grass	360	Dried grass	360	Dried grass	360	19. x. 50 and 31. x. 50
15, 16	716	Silage	1455	Silage	1455	Silage	1455	19. x. 50 and 31. x. 50

* Only on this diet for 6 days previous to experiment.

Table 2. *Composition of feeding stuffs (see Table 1 and text)*

Feeding stuff	N (Kjeldahl) (%)	Dry matter (%)	N as % of dry matter
Hay I*	1·35	87·8	1·54
Hay II*	1·37	89·2	1·54
Concentrate I†	2·61	85·7	3·04
Concentrate II‡	2·35	86·2	2·73
Frozen grass§	0·63	18·7	3·37
Dried grass‖	3·02	86·8	3·48
Silage¶	0·59	16·0	3·69
Casein**	12–13	85–90	—

* Mixed rye grass-clover hays from Aberdeenshire.

† 5 parts ground maize, 2 parts wheat bran, 2 parts crushed oats, 1 part linseed-cake meal and 1 part white-fish meal.

‡ 8 parts ground maize, 2 parts wheat bran and 2 parts crushed oats.

§ A grass-clover mixture cut at the Duthie-Craibstone Farm in May 1950 and stored at −20°.

‖ The same grass-clover mixture dried commercially.

¶ The same grass-clover mixture chopped and ensiled in small-scale concrete silos without addition.

** Various preparations by commercial firms.

of commercial *n*-valeric and '*iso*valeric' acids. Fig. 6, for comparison, shows an analysis of a made-up mixture of acetic, propionic, *iso*butyric, *n*-butyric, '*iso*valeric', *n*-valeric, γ-methylvaleric ('*iso*caproic')· and hexanoic acids. From these experiments the predominance of branched-chain C_5 acids and the low concentration of C_6 acids in

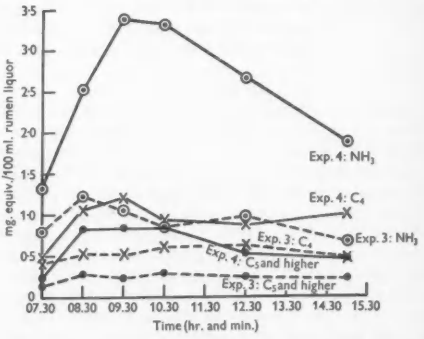

Fig. 1. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding concentrates (07.30 hr.) and hay (11.00 hr.) (Exp. 3, – – – –), and concentrates with casein (07.30 hr.) and hay (11.00 hr.) (Exp. 4, ——).

rumen liquor is apparent. The forms of the curves leave no doubt as to the identities of the C_4 and lower VFA's.

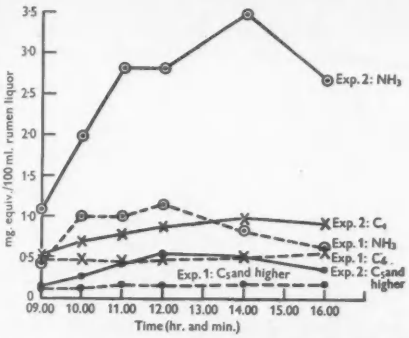

Fig. 2. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding concentrates with casein (09.00 hr.) and hay (11.00 hr.) (Exp. 2, ——), and hay only (09.00 hr. and 11.00 hr.) (Exp. 1, – – – –).

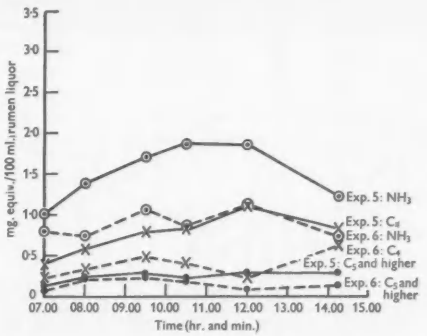

Fig. 3. Concentrations of ammonia and higher VFA's in rumen liquor of sheep after feeding fresh grass (07.00 and 11.00 hr.). (Exp. 5, ——; Exp. 6, – – – –.)

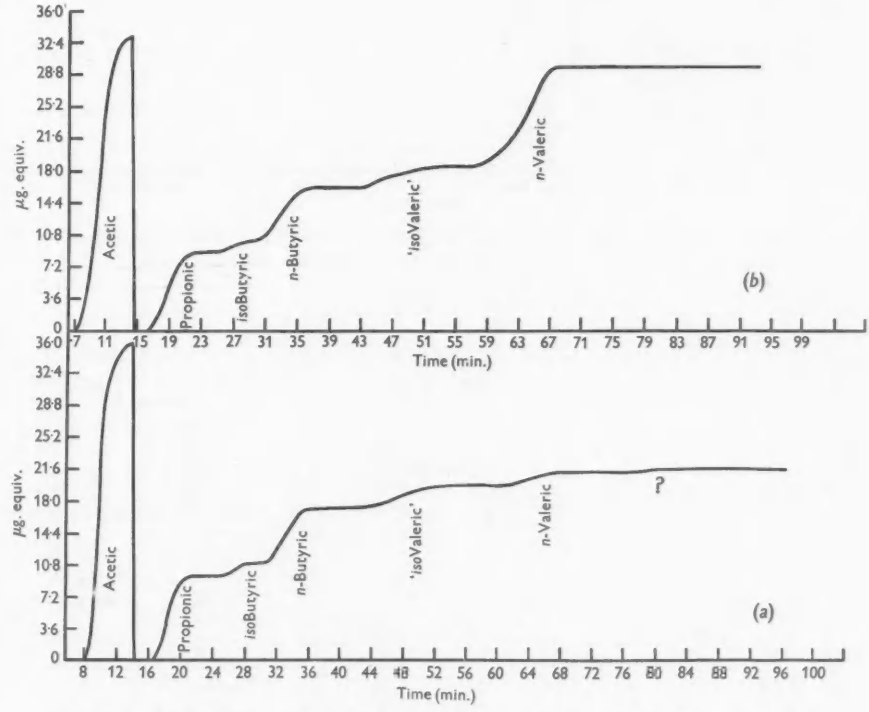

Fig. 4. Gas-liquid partition chromatogram of VFA's from rumen liquor of sheep (*a*) without and (*b*) with addition of *n*-valeric acid.

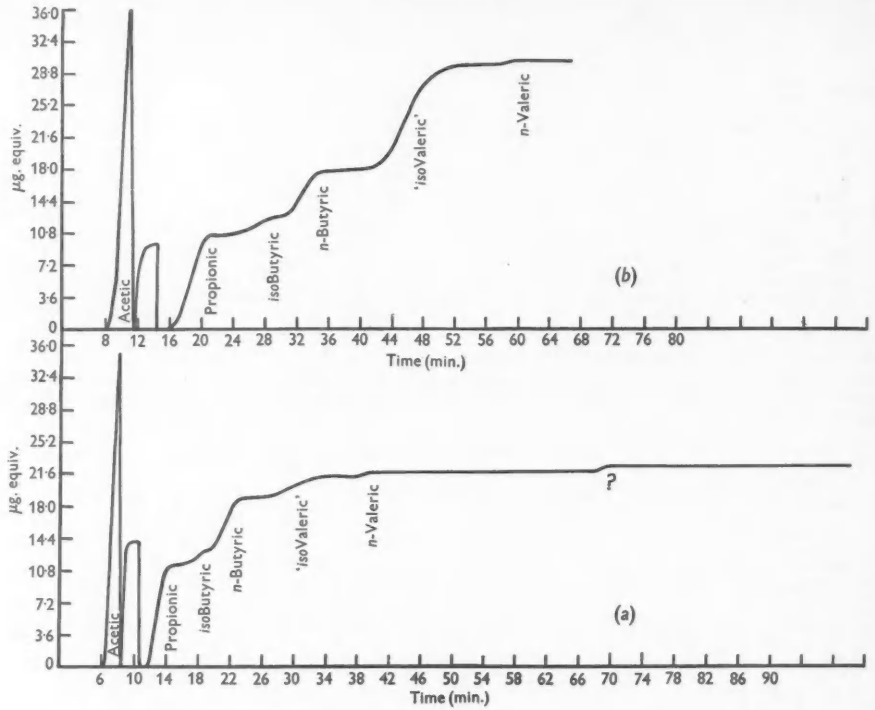

Fig. 5. Gas-liquid partition chromatogram of VFA's from rumen liquor of sheep (*a*) without and (*b*) with addition of commercial '*iso*valeric' acid.

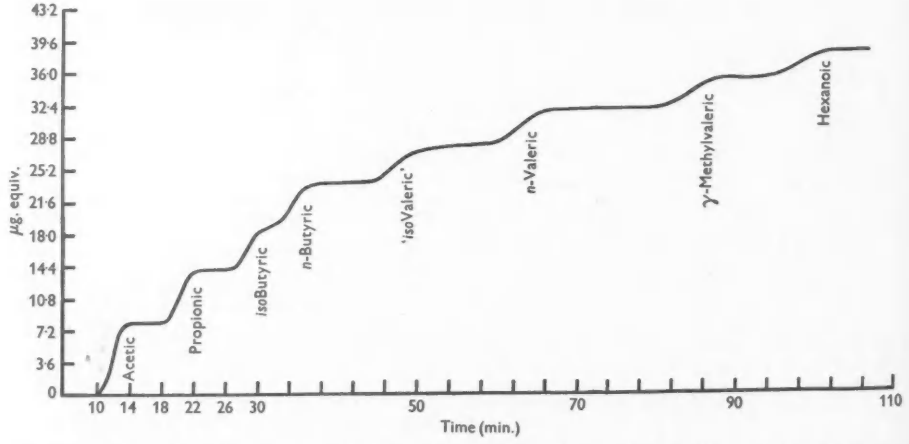

Fig. 6. Gas-liquid partition chromatogram of a mixture, in roughly equal proportions, of commercial acetic, propionic, *iso*butyric, *n*-butyric, '*iso*valeric', *n*-valeric, γ-methylvaleric ('*iso*caproic') and hexanoic acids.

Analyses of changes of volatile fatty acid composition of rumen liquor on feeding

In Exps. 7–16 the evolution of ammonia was followed during the day (Chalmers & Synge, to be published). Curves for this are given in Figs. 7 and 8. Larger samples of rumen liquor were taken for VFA analysis by the method of James & Martin at 7 a.m. immediately before feeding and at times (indicated by the arrows on the ammonia curves) when it was hoped that the ammonia concentration would be high. The results of these analyses are set out in Table 3. The large absolute and relative increases of the branched-chain isomers when casein

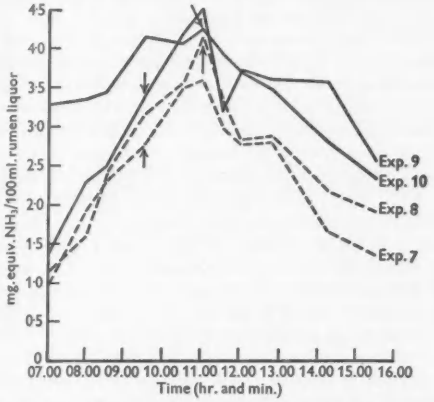

Fig. 7. Time course of ammonia concentration in rumen liquor of sheep fed casein and concentrate at 07.00 hr. and hay at 11.00 hr. Samples for analysis of VFA's were taken before feeding and at the times indicated by the arrows on the curves.

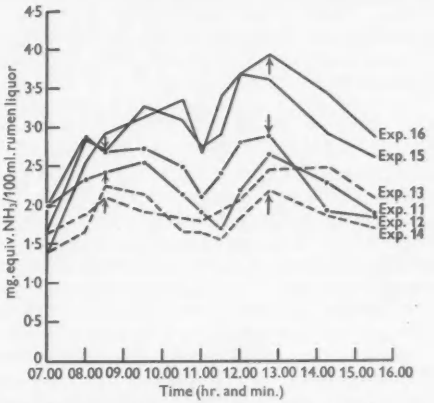

Fig. 8. Time course of ammonia concentration in rumen liquor of sheep fed (at 07.00 and 11.00 hr.) frozen grass (—·—·), dried grass (– – – –) and silage (——). Samples for analysis of VFA's were taken before feeding and at the times indicated by the arrows on the curves.

Table 3. *Changes in concentrations of volatile fatty acids in rumen liquor of sheep on different diets*

(For details of animals, feeding, times of sampling, and concurrent ammonia concentrations see Tables 1 and 2, Figs. 7 and 8 and text.)

Exp. no.	Feed		Total VFA's (mg. equiv./ 100 ml.)	Individual VFA's as % of total VFA (molecular basis)						
				Acetic	Propionic	*iso*-Butyric	*n*-Butyric	Branched C_5	*n*-Valeric	C_6 etc.
7	Concentrate + 50 g. casein	Before	5·6	64·4	18·0	2·8	7·7	1·2	0·9	Trace
		After	11·8	56·9	23·7	1·5	8·5	3·9	1·4	Trace
8	Concentrate + 50 g. casein	Before	7·3	65·5	22·9	1·4	10·6	2·7	1·0	Trace
		After	14·4	45·5	23·5	2·0	10·6	3·5	2·3	Trace
9	Concentrate + 100 g. casein	Before	8·6	60·0	14·3	1·3	10·2	2·1	1·0	0·8
		After	10·6	67·0	18·5	2·5	11·7	5·0	2·4	1·9
10	Concentrate + 100 g. casein	Before	10·6	55·7	20·6	1·7	19·3	3·1	2·3	Trace
		After	16·0	53·1	21·7	2·7	16·6	3·6	3·3	Trace
11	Frozen grass	Before	8·6	65·7	17·1	2·1	9·3	3·1	1·3	0·4
		After	11·6	54·0	22·6	1·7	14·2	1·1	1·2	Trace
12	Frozen grass	Before	5·8	67·2	17·1	2·2	9·3	2·9	1·6	Trace
		After	13·8	55·2	24·3	1·2	12·5	1·3	1·6	Trace
13	Dried grass	Before	9·8	69·5	18·6	1·4	8·1	1·9	0·7	Trace
		After	14·9	57·7	22·2	0·8	8·4	1·4	0·8	Trace
14	Dried grass	Before	9·4	55·5	15·7	1·0	7·6	1·3	1·1	Trace
		After	13·5	63·5	26·0	0·5	11·9	0·8	1·5	Trace
15	Silage	Before	5·5	70·0	16·2	2·6	8·6	3·2	0·6	0·9
		After	11·0	53·2	25·4	1·3	7·4	2·5	1·7	0·8
16	Silage	Before	7·5	72·6	14·9	1·6	7·7	1·9	1·0	Trace
		After	14·5	66·6	23·6	1·5	10·7	2·7	2·3	Trace
Analysis of silage fed in Exps. 15 and 16			10·6	74·0	10·7	Nil	5·1	Nil	1·1	Nil

was fed are apparent. With the grass products there is a relative fall and little absolute increase in the concentrations of branched-chain VFA's, except on feeding silage where there is a substantial absolute increase. This may be correlated with the higher concentrations of ammonia found on feeding the silage (Fig. 8). The parallel analysis of the VFA's of the silage fed (Table 3, last entry; cf. Brouwer & Nijkamp, 1950) shows that little or no preformed branched-chain VFA's were present. They have, however, been reported in certain silages by Brahm (1925, 1927).

DISCUSSION

It is assumed that αα-dimethylpropionic (trimethylacetic, pivalic) acid, which has never so far been found in nature, is absent from rumen contents. This is not readily separated from *n*-butyric acid by the methods used. It follows that *iso*butyric, *n*-butyric, '*iso*valeric' (α-methylbutyric and/or *iso*valeric) and *n*-valeric acids occurred in the rumen contents of the sheep studied; C_6 homologues, if present, occurred only in traces. It has for some time been recognized that volatile fatty acids other than acetic, propionic and *n*-butyric acids are present (cf. especially Elsden, 1946), but the demonstration of their nature has now been made simple by use of the technique of James & Martin (1952).

Since Elsden & Phillipson (1948) reviewed knowledge of these acids in rumen contents, Schambye & Phillipson (1949) determined 'butyric plus higher' acids, and Reid (1950) found in addition to acetic 'small amounts of propionic, butyric and at least one other acid' in the systemic blood of the sheep. A more detailed study has been made by McClymont (1951) who determined higher VFA's in the rumen of oxen fed on various diets. C_4 isomers ranged from 10·4 to 18·1 % of total VFA's and (C_5 and higher) from 2·6 to 5·1 % (molecular basis). In a special study of the latter fraction (diet not specified) C_5 isomers accounted for 51 % and C_6 for 38 %. Since the present work was done Gray, Pilgrim, Rodda & Weller (1951) have published a preliminary account of a detailed study of the VFA's from sheep rumen contents. The main fractionation was by unspecified partition-chromatographic procedures. A sheep fed wheaten hay gave *n*-butyric acid, 9·0; *iso*butyric acid, 0·7; C_5 acids, 2·6; C_6 acids, 0·7 %; C_7 (?), trace. It is not clear whether these percentages are on a molecular or weight basis, but their figure for *iso*-butyric acid, determined by 'a separate method', is somewhat lower in relation to *n*-butyric acid than was usually found in the present work. Gray *et al.* (1951) also found by comparing R values that a greater proportion of the C_5 acids were *n*-valeric than were branched-chain acids. Since this is the opposite of what was usually found here, and since comparing R values is a much less reliable method of identification than the running of mixed chromatograms with natural and synthetic materials, a decision must await the publication by Gray and colleagues of fuller data.

Discussion of the origin and fate of these acids of the rumen is deferred to the following paper, where these problems are considered in relation also to the *in vitro* data there recorded. Although branched-chain C_4 and C_5 VFA's are well known constituents of certain plants and have been widely recognized as fermentation and putrefaction products derived from proteins by the action of micro-organisms, there have been surprisingly few observations of their occurrence in animals, except in intestinal worms (Flury, 1912; Epps, Weiner & Bueding, 1950) and, esterified, in the fat of porpoises and whales (e.g. Lovern, 1934). Brieger (1877) obtained evidence for the occurrence of *iso*butyric acid (as well as acetic and *n*-butyric acids) in human faeces, and Klinc (1935) found it in human urine. The present work establishes that, in the rumen of the sheep, a significant proportion of the C_4 VFA's and the greater part of the C_5 VFA's have branched-chain structures and demonstrates a parallelism in the changes of concentrations of ammonia and of the C_4 and C_5 acids in the rumen after administration of protein-rich foods. The only easy interpretation of these facts seems to be that suggested above, namely that a principal source of both the ammonia and the acids is deaminative attack by micro-organisms on the amino-acids of the protein fed.

SUMMARY

1. Using the gas-liquid partition chromatogram of James & Martin (1952) it was found that branched-chain lower fatty acids occur in the rumen of the sheep on a variety of diets. A significant proportion of the C_4 acids was *iso*butyric acid, and branched-chain isomers often made up the greater part of the C_5 acids. C_6 acids were present only in small amounts.

2. The concentrations of ammonia and of the various volatile fatty acids in the rumen were determined at different times after feeding.

3. Increased ammonia concentration was correlated with increased concentrations of *iso*butyric acid and of the C_5 acids, which are therefore considered to arise from microbial attack on protein in the rumen.

I am very grateful to Dr A. T. James and Dr A. J. P. Martin, F.R.S., for instruction in the use of their gas-liquid partition chromatogram, and to Mr R. S. Reid and Mr G. Masson for constructing the necessary apparatus.

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Degradation of Protein in the Rumen of the Sheep

2. THE ACTION OF RUMEN MICRO-ORGANISMS ON AMINO-ACIDS

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In the previous paper (el-Shazly, 1952) evidence that ammonia is formed from protein in the rumen was reviewed and it was suggested that some of the volatile fatty acids (VFA's) found in the rumen originate mainly from the breakdown of protein. To investigate more closely the character of this breakdown the action of rumen micro-organisms on free amino-acids has now been studied. Only low concentrations of free amino-acids exist in the rumen liquor, but there is evidence (Sym, 1938) of protease activity by the micro-organisms; McDonald (1948) has measured the disappearance of zein from the rumen and Chalmers & Synge (to be published) have observed the dissolution of lumps of denatured casein. The nature of this proteolytic action, which is likely to prove of great importance for the protein economy of the animal, must await more detailed study. The present work has been concerned solely with the fate of the free amino-acids which it is reasonable to assume are liberated in the proteolysis.

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The best course would have been to study the fate of amino-acids added in low concentrations to whole rumen liquor within a very short time of its removal from the animal, but this was not technically feasible. It was, however, possible to observe the disappearance of somewhat high concentrations of amino-acids added to freshly removed rumen liquor during anaerobic incubation for a few hours; the high concentrations of ammonia and VFA's already present prevented detailed study of new formation of these products from the added amino-acids. However, following recommendations of Dr S. R. Elsden, conditions were found for using washed suspensions of mixed rumen micro-organisms which attacked amino-acids present in mixtures at much the same relative rates as these were attacked in whole rumen liquor. Under these circumstances it was relatively easy to determine the end products of the fermentation and to construct a rough balance sheet for the reactions. Although the reactions may take a different course under physiological conditions (where the amino-acid concentrations are lower, other essential nutrients for the micro-

organisms are present in greater variety and other fermentations, e.g. of carbohydrate, may be proceeding) the results with washed suspensions probably give a fair idea of the physiological fate of part of the protein destroyed in the rumen.

Many workers have studied the attack by micro-organisms on amino-acids administered singly. For the present purpose this did not seem to be a good approach, since under physiological conditions many different amino-acids must become available to the micro-organisms simultaneously. Moreover, as Stickland (1934) first showed, interactions between amino-acids may completely modify the course of fermentation. Such interaction was, in fact, demonstrated in the course of the present work; accordingly, for most of the experiments an acid hydrolysate of casein was used to supply the mixed amino-acids. This protein was chosen because much of the work of my colleagues Dr M. I. Chalmers, Dr D. P. Cuthbertson and Dr R. L. M. Synge has involved the administration of casein supplements to sheep.

EXPERIMENTAL

Materials and general methods

The mixture of amino-acids usually employed was a casein hydrolysate (vitamin-free; Ashe Laboratories Ltd., London). Filter-paper chromatography of amino-acids was as described by Synge (1951*b*) using phenol/collidine but omitting H_2O_2 unless otherwise specified. NH_3 was determined as in the previous paper (el-Shazly, 1952).

Behaviour of free amino-acids on incubation with rumen contents

Rumen-liquor samples, collected from sheep on a diet of casein, concentrates and hay (el-Shazly, 1952; Table 1, Exp. 2) in the morning before feeding, were strained through muslin and saturated with CO_2. Four 20 ml. portions were then taken in boiling tubes *A*, *B*, *C* and *D*. Tubes *C* and *D* contained casein hydrolysate (8·1 mg. N) previously evaporated to dryness. After mixing, a layer of liquid paraffin B.P. was pipetted on to the contents of all four tubes; tubes *B* and *D* were incubated for 3 hr. at 40°. Tubes *A* and *C* were analysed forthwith. Analysis of the aqueous phase from all four tubes was as follows:

After cooling to 0°, 10 ml. portions were dialysed in washed cellophan tubing for 24 hr. at 0° against 30 ml. of water. NH_3 was determined on portions of the diffusates. Other portions were acidified to pH 1 with H_2SO_4, steam-distilled to remove VFA's and desalted according to Consden, Gordon & Martin (1947); two-dimensional filter-paper chromatograms of the amino-acids present were then prepared.

The NH_3 determinations showed increases of NH_3-N (differences between tubes *B* and *D*) on incubation of about 15–25 mg./100 ml. rumen liquor taken, i.e. 35–65% of the N of the casein hydrolysate appeared as NH_3; these figures are of necessity rough owing to the considerable amounts of NH_3 initially present and formed during incubation without added casein hydrolysate. The filter-paper chromatograms from tubes *A* and *B* showed only faint traces of amino-acids. All those of the original casein hydrolysate were seen with *C*, while with *D* it was seen that phenylalanine, tyrosine, and proline had completely disappeared; alanine, threonine, glycine, serine, and aspartic acid were very weak or absent; glutamic acid was considerably weakened while the remaining spots were relatively strong, although obviously weaker than the corresponding spots from *C*.

A new spot always appeared, corresponding in position and colour on the phenol/collidine chromatograms with γ-aminobutyric acid, δ-aminovaleric acid, etc. Using phenol/*n*-butanol-acetic acid chromatograms (cf. Synge, 1951*a*) this spot corresponded with δ-aminovaleric acid, and γ-aminobutyric acid, etc., were thus shown to be absent (mixed chromatograms with authentic materials). On incubating rumen liquor as above, but with only L-proline (7·4 mg.) added, there was no obvious disappearance of proline, nor was any δ-aminovaleric acid formed. On incubation with L-proline (7·4 mg.) and DL-alanine (8·3 mg.), the alanine spot was weakened, the proline spot almost disappeared and a strong spot corresponding with δ-aminovaleric acid was produced. On incubation with the same amount of DL-alanine alone, the alanine spot was weakened but no new spot appeared.

In the course of these experiments indications were obtained that some added material or reaction product inhibited formation of NH_3, since if the amounts of casein hydrolysate added to the rumen liquor were increased on that stated above, the proportion of deamination on incubation was diminished. Control experiments showed that this effect was not due to NH_3.

Isolation and identification of δ-aminovaleric acid formed by Stickland reaction (cf. Stickland, 1935; Woods, 1936). Rumen liquor from a sheep on a similar diet (el-Shazly, 1952; Table 1, Exp. 7), collected before feeding, was strained through muslin and 400 ml. were incubated with 127 mg. of L-proline and 140 mg. of DL-alanine at 40° for 3 hr. in a closed vessel; N_2 containing 5% (v/v) of CO_2 was bubbled through. The mixture was dialysed in cellophan tubing against 2 l. of water for 48 hr. at 0°. The diffusate was acidified to pH 1 with H_2SO_4, concentrated to small volume *in vacuo* and then steam-distilled to complete removal of VFA's. $Ba(OH)_2$ was added to bring the residue to pH 4. $BaSO_4$ was removed by filtration and the filtrate was desalted in the apparatus of Consden *et al.* (1947). A filter-paper chromatogram showed that proline had completely disappeared, being replaced by a strong spot in the position of δ-aminovaleric acid. Small amounts of alanine, valine, leucine, phenylalanine, etc., were present. The preparation was evaporated to dryness *in vacuo* and fractionated on a cellulose-powder chromatogram (140 g., 5·4 cm. diam.) using the system *n*-butanol-water-acetic acid (4:5:1 by vol.) as for the isolation of γ-aminobutyric acid (Synge, 1951*a*). The effluent fractions containing δ-aminovaleric acid were pooled and evaporated to dryness *in vacuo*; the residue was dissolved in water and on filtration and evaporation yielded 205 mg. of a clear gum. Two successive crystallizations from ethanol-ether yielded 85 mg. of pale-yellow crystals having m.p. 154° (uncorr.). The product did not, on admixture, depress the m.p. (156–158°) of authentic δ-aminovaleric acid prepared according to Wallach (1900) from *cyclo*pentanone oxime (cf. Fox, Dunn & Stoddard, 1941). In two-dimensional filter-paper chromatograms (phenol/*n*-butanol-acetic acid) the material gave a single

spot with or without admixture of authentic δ-aminovaleric acid. $[\alpha]_D^{22.5°}$ +1° in water (*l*, 0·5; *c*, 7·0). (Found: C, 50·4; H, 9·2; N (Kjeldahl), 11·7. Calc. for $C_5H_{11}O_2N$: C, 51·3; H, 9·4; N, 11·9%.) In the Van Slyke amino-N reaction (30 min. at 21°) 94% of the N reacted (for previous data see Synge, 1951*a*). In the ninhydrin-CO_2 reaction according to Van Slyke, Dillon, MacFadyen & Hamilton (1941), using citrate buffer (pH 2·5), the result was completely negative. A very slight reaction in the amino-N determination according to Pope & Stevens (1939) was given equally by the synthetic material. The isolated material behaved in electrical migration experiments as an ω- rather than an α-amino-acid (cf. Synge, 1951*a*).

with and without casein hydrolysate as described above except that N_2 (freed from O_2 by passing over hot Cu) rather than liquid paraffin was used for securing anaerobic conditions. NH_3 was determined before and after incubation. The results (Table 1) show close agreement in the amounts of NH_3 produced from the amino-acids added. Filter-paper chromatograms were likewise run on the incubated mixtures. With the washed suspensions phosphate was removed by precipitation in the presence of $CaSO_4$ and NaOH before desalting; the steam distillation was omitted as only small amounts of VFA's were present. The chromatograms likewise showed closely similar results in the extent to which the various amino-acids were attacked, the only noticeable

Table 1. *Formation of ammonia from amino-acids by rumen liquor and by washed suspensions derived therefrom*

(For details see text. Exps. 1 and 2 were with rumen liquor obtained on different days from a sheep on a casein-concentrate-hay diet (el-Shazly, 1952; Table 1, Exp. 2).)

	Ammonia N (mg./100 ml.)			
	Before incubation	After incubation	Difference	Difference attributable to casein hydrolysate
Exp. 1				
Rumen liquor	14·7	27·6	12·9	
Rumen liquor + casein hydrolysate (81 mg. N/100 ml.)	16·4	57·4	41·0	28·1
Washed suspension	2·8	6·2	3·4	
Washed suspension + casein hydrolysate (81 mg. N/100 ml.)	2·6	29·7	27·1	23·7
Exp. 2				
Rumen liquor	14·2	19·7	5·5	
Rumen liquor + casein hydrolysate (40 mg. N/100 ml.)	15·8	40·9	25·1	19·6
Washed suspension	2·0	4·7	2·7	
Washed suspension + casein hydrolysate (40 mg. N/100 ml.)	3·5	20·7	17·2	14·5

Experiments with washed suspensions of micro-organisms from rumen liquor

Preparation of washed suspensions. Use of simple buffer solutions during centrifugation gave preparations having very low deaminating power. The addition of Na_2S as recommended by Dr S. R. Elsden (cf. Johns, 1951) gave, however, satisfactory preparations. Rumen-liquor samples, collected from the sheep before feeding in the morning, were cooled to 0°, strained through muslin, centrifuged very lightly to remove plant particles and Protozoa, and then centrifuged 15 min. at 17000 rev./min. on the high-speed attachment head (previously cooled to below 0°) of a 'MSE Major' centrifuge (Measuring and Scientific Equipment Ltd., London S.W. 1). The resulting precipitate was resuspended to the original volume with 0·033 M-potassium phosphate buffer (pH 6·5) containing 0·02% (w/v) $Na_2S.9H_2O$. Centrifugation was repeated and the precipitate was resuspended with the same potassium phosphate buffer (without added Na_2S), and used immediately for the incubation experiments as described below.

Comparison of attack on amino-acids by washed suspensions and by rumen liquor. Washed suspensions, and the rumen liquor from which they had been prepared, were incubated

Table 2. *Formation of ammonia from amino-acids by washed suspensions from the rumen of sheep on different diets*

(Portions (5 ml.) of washed suspension incubated with casein hydrolysate (1·8 mg. N) for 3 hr. at 40° under nitrogen.)

	Ammonia N (mg./100 ml.)		
	Sheep 1	Sheep 2	Sheep 3
(*a*) Diet: casein, concentrates, hay:			
Before incubation	1·5	2·2	3·3
After incubation	17·6	35·4	30·2
Increase	16·1	33·2	26·9
(*b*) Diet: hay only:			
Before incubation	2·9	2·6	2·8
After incubation	6·2	5·2	4·0
Increase	3·3	2·6	1·2
(*c*) Diet: fresh grass only:			
Before incubation	—	3·1	3·3
After incubation	—	25·2	23·3
Increase	—	22·1	20·0

difference being that glutamic acid was not much attacked by the washed suspensions.

Deaminating power of washed suspensions from sheep on different diets. Three sheep received during successive 3-week periods diets as indicated by el-Shazly (1952; Table 1). (*a*) A concentrate-casein-hay diet (as in Exps. 7 and 8); (*b*) an all-hay diet (as in Exp. 1); (*c*) a fresh-grass diet (as in Exps. 5 and 6) and had rumen-ammonia curves similar to those recorded. At the end of each 3-week period deamination on incubation of casein hydrolysate with washed suspensions as described above was studied, with the results shown in Table 2.

Quantitative study of reaction products formed from casein hydrolysate by washed suspensions of rumen micro-organisms

Attempts were made to construct balance sheets for the fermentations studied. Determinations were made, before and after incubation, of: (*a*) ammonia, as described above; (*b*) α-amino-acid N of free amino-acids by the ninhydrin-CO_2 procedure (Van Slyke *et al.* 1941) using citrate buffer at pH 2·5; (*c*) ether-extractable acids and VFA's by the techniques used by el-Shazly (1952); (*d*) gaseous exchanges during incubation, in Warburg manometers as described below. Not all determinations were done in each experiment. Rumen-liquor samples were from animals on a casein-concentrate-hay diet as above, taken before feeding.

Exp. 1. Portions of washed suspension (7·5 ml.) were incubated with casein hydrolysate (2·83 mg. N) for 3 hr. at 40° under N_2; the micro-organisms were then centrifuged down at high speed and suitable portions of the clear supernatants analysed. Parallel analyses were done on unincubated mixture.

Exps. 2–6. These were done with 2·7 ml. portions of washed suspension incubated with casein hydrolysate (2·6 mg. N dissolved in 0·3 ml. of the same phosphate buffer) in Warburg manometers under N_2 or A. With KOH solution in the centre compartment to absorb CO_2 the course of

Table 3. *Balance sheet of action of washed suspensions on casein hydrolysate*

(Casein hydrolysate incubated at 40° for times shown. For experimental details see text.)

	mg. atoms or m-moles/100 ml. suspension		
	Before incubation	After incubation	Change
Exp. 1 (incubation time 3 hr.):			
α-Amino-acid N	2·03	0·60	−1·43
NH_3	0·47	1·48	+1·01
VFA's	0·31	0·88	+0·57
Non-volatile ether-extractable acids (mg. equivs.)	0·09	0·07	−0·02
Exp. 2 (incubation time 1·75 hr.):			
α-Amino-acid N	1·99	1·68	−0·31
NH_3	0·11	0·43	+0·32
CO_2	—	—	+0·30
Exp. 3 (incubation time 1·75 hr.):			
α-Amino-acid N	1·98	1·54	−0·44
NH_3	0·09	0·56	+0·47
Total ether-extractable acid (mg. equivs.)	0·37	0·74	+0·37
CO_2	—	—	+0·39
Exp. 4 (incubation time 1·75 hr.):			
α-Amino-acid N	1·65	1·36	−0·29
NH_3	0·09	0·40	+0·31
Total ether-extractable acid (mg. equivs.)	0·07	0·25	+0·18
CO_2	—	—	+0·35
Exp. 5 (incubation time 2 hr.):			
NH_3	0·11	0·79	+0·68
VFA's	0·41	1·03	+0·62
CO_2	—	—	+0·56
Exp. 6 (incubation time 2 hr.):			
NH_3	0·13	0·59	+0·46
VFA's	0·30	0·73	+0·43
CO_2	—	—	+0·49

Table 4. *Volatile fatty acids formed on incubation of casein hydrolysate with washed suspensions of rumen micro-organisms*

(Figures in brackets indicate the percentage of total increase contributed by each acid.)

	mg. equiv./100 ml.								
	Exp. 1 (Table 3) (Elsden technique)			Exp. 5 (Table 3) (James & Martin technique)			Exp. 6 (Table 3) (James & Martin technique)		
Acid	Before incubation	After incubation	Increase	Before incubation	After incubation	Increase	Before incubation	After incubation	Increase
Acetic	0·263*	0·680*	0·417* (73·4%)	0·278	0·461	0·183 (29·5%)	0·117	0·450	0·333 (78·5%)
Propionic	0·018	0·080	0·062 (10·9%)	0·053	0·356	0·303 (48·9%)	0·097	0·143	0·046 (10·8%)
*iso*Butyric	0·010	0·055	0·045 (7·9%)	0·000	0·030	0·030 (4·8%)	0·000	0·007	0·007 (1·6%)
n-Butyric				0·033	0·100	0·067 (10·8%)	0·048	0·067	0·019 (4·5%)
Branched C_5	0·022	0·066	0·044 (7·7%)	0·020	0·050	0·030 (4·8%)	0·018	0·034	0·016 (3·8%)
n-Valeric				0·025	0·045	0·020 (3·2%)	0·022	0·018	— —
Total	0·313	0·881	0·568	0·410	1·030	0·620	0·300	0·725	0·425 —

* By difference.

the reaction (judged by amino-acid consumption and NH_3 production) was no different from that when CO_2 was allowed to accumulate. No significant output of H_2 or CH_4 or uptake of N_2 or A was observed. CO_2 output was determined by measuring the difference in gas output in manometers (not having KOH) on tipping in 0·2 ml. 10 N-H_2SO_4 from the side arms at the beginning and end of incubation respectively.

The results are given in Table 3. In general the rough equivalence of moles ammonia, carbon dioxide and VFA's produced to moles amino-acid destroyed is striking.

Distribution of volatile fatty acids. In Exps. 1, 5 and 6 the distribution of VFA's was ascertained chromatographically (for techniques see el-Shazly, 1952), and is given in Table 4. It is seen that, while acetic and propionic acids predominate, the proportions of C_5 isomers present tend to exceed those usually found in rumen liquor.

DISCUSSION

No information has yet been gained as to which of the micro-organisms are responsible for bringing about the decompositions observed. The present experiments show, however, that the power of mixed rumen organisms to decompose amino-acids to ammonia and other products is strikingly increased by increasing the content of soluble protein in the diet. On microscopic examination of the different populations Miss Marjorie Masson found no correspondingly striking changes in the morphology or staining properties of the micro-organisms present. The very slight deamination obtained with washed suspensions from hay-fed animals serves as a control, suggesting that selective growth of deaminating organisms during the incubations probably did not intervene to alter greatly the proportions in which the organisms occurred from those existing in the rumen.

The chemical changes resulted mainly in the formation of VFA's, ammonia and carbon dioxide. The virtual absence of hydrogen, gaseous hydrocarbons and other ether-extractable organic acids was demonstrated. Formation of alcohols and neutral carboxyl compounds was not excluded. The only other nitrogenous fermentation product detected was δ-aminovaleric acid, which was shown experimentally to arise from proline (cf. Stickland, 1934, 1935) and may also have arisen from arginine via ornithine (Woods, 1936). The reaction products observed were, in fact, strikingly similar to those reported by previous workers for the anaerobic decomposition of individual amino-acids by a variety of micro-organisms. Cohen (1949) has given an excellent review of this subject, in which the experimental evidence of the importance of mutual oxidation-reduction reactions of the Stickland type is set out. Such a reaction between alanine and proline was demonstrated in the present work, and the resulting δ-aminovaleric acid was isolated and characterized. Further, from the direct evidence obtained by the procedure of James & Martin (1952) that branched-chain C_4 and C_5 VFA's are formed, it is reasonable to postulate that these arise from valine, leucine and isoleucine acting as hydrogen donors in reactions similar to those studied in detail by Cohen, Nisman and their colleagues at the Institut Pasteur (Cohen-Bazire, Cohen & Prévot, 1948*a*, *b*; Nisman, Raynaud & Cohen, 1948*a*, *b*; Nisman & Thouvenot, 1948; Nisman & Vinet, 1949*a*, *b*, 1950; Rosenberg & Nisman, 1949*a*, *b*; Saissac, Raynaud & Cohen, 1948).

It is only in relatively few cases that evolution of hydrogen has been detected in amino-acid fermentations (cf. Woods & Clifton, 1937, 1938; Woods & Trim, 1942; Cardon & Barker, 1947). In the present fermentations of amino-acid mixtures by mixed organisms the availability of readily reduced amino-acids such as glycine and proline may have encouraged reactions of the Stickland type and prevented the appearance of molecular hydrogen or methane as reaction products. Different reducible substances may well also operate when carbohydrate is being simultaneously fermented.

The rapid and complete disappearance of tyrosine and phenylalanine should be noted. The present data do not make it clear whether they were assimilated or fermented by the micro-organisms, but it is interesting that Cohen (1949) reports a complete absence of data on anaerobic fermentation of phenylalanine or lysine. The latter did not rapidly disappear under the conditions of the present work.

The predominance of acetic acid among the resulting VFA's is notable. *n*-Butyric acid results in lower proportion than is normally present in the VFA's of rumen liquor. Propionic acid and *n*-valeric acid (which could conceivably arise from arginine, ornithine, proline, δ-aminovaleric acid or lysine) seem to be variable, while the branched-chain C_4 and C_5 acids are in higher proportion. These, whose probable origin from valine, leucine and isoleucine has already been discussed, are formed in fair quantity despite the relatively slow attack by the micro-organisms on these amino-acids. It is these branched-chain acids which, as previously shown (el-Shazly, 1952), are characteristic products of the breakdown of protein in the rumen under physiological conditions. The relative amounts of these acids that are absorbed from the rumen may prove to be in excess of the proportion suggested by their relative concentration in rumen liquor (cf. Johnson, 1951; Pennington, 1951). In any case their absorption and metabolic fate seem worthy of study.

Their carbon skeletons, unlike those of the corresponding α-keto acids, are probably not available for resynthesis of the corresponding 'essential' amino-acids within the mammalian body; the loss of dietary nitrogen absorbed as ammonia is thus made more certain. The branched-chain VFA's are presumably oxidized in the animal body. Considerable evidence has accumulated that they may be ketogenic; whilst there is some controversy about *iso*butyric and α-methylbutyric acids, all workers seem to be agreed on the ketogenicity of *iso*valeric acid (see Cohen, 1937; Wick, 1941; Bloch, 1944; Valdiguié & Séguélas, 1946; Atchley, 1948; Grafflin & Green, 1948; Coon, 1950; Gray, Adams & Hauptmann, 1950; Zabin & Bloch, 1950). It does not therefore seem unreasonable to postulate that branched-chain VFA's may play a part in at least some of those forms of ketosis in ruminants that have been attributed to high-protein diets. Should this speculation be substantiated, a number of possible courses for the control of these conditions would suggest themselves.

Note

It is of interest that, since these papers were written, Hansen & Shorland (1951) have described the isolation from the higher fatty acids of butter fat of branched-chain C_{17} acids which they considered to be 14-methyl- and 15-methyl-hexadecanoic acids. These could arise by successive condensation of C_2 units on to the carboxyl ends of the branched-chain C_5 acids recognized in the present work (cf. Popják, French, Hunter & Martin, 1951).

SUMMARY

1. The amino-acids of an acid hydrolysate of casein were attacked under anaerobic conditions at similar relative rates by sheep rumen contents and by washed suspensions of micro-organisms therefrom.

2. L-Proline was reduced when other amino-acids were present; the resulting δ-aminovaleric acid was isolated and characterized (cf. Stickland, 1934, 1935).

3. Using washed suspensions, the main reaction products from casein hydrolysate were ammonia, carbon dioxide and volatile fatty acids in roughly equimolecular proportions; there was no significant formation of hydrogen, methane or non-volatile organic acids. The volatile fatty acids were straight- and branched-chain C_2 to C_5 acids.

4. The power of washed suspensions of rumen micro-organisms to deaminate amino-acids depended on the diet of the animal.

5. The probable origin of the branched-chain acids by Stickland reactions from valine, leucine and isoleucine, and the nutritional significance of the deamination reactions for the animal are discussed.

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The Composition of the Honeydew Excreted by Australian Coccids of the Genus *Ceroplastes*

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The coccids included in the genus *Ceroplastes* are popularly known as 'wax scales' from the large amount of waxy secretion covering the adult female. Froggatt (1921) recognized three species as occurring in Australia, namely, *C. ceriferus* And., *C. rubens* Marshall and *C. rusci* L. A fourth species, *C. floridensis* Comst., is recorded in the catalogue of Fernald (1903) as occurring in Australia, and Froggatt remarks that if this is the case it is a rare and comparatively unknown coccid. Later (Zeck, 1932) it was shown that another species, *C. destructor* Newstead, is of wide distribution and that it was formerly confused with *C. ceriferus*. All these coccids are considered to be introduced species, although *C. rubens* was originally described from Australia (Froggatt, 1921).

These wax scales are pests and occur in tropical and subtropical regions. *C. ceriferus* (Indian white wax scale), although widely distributed, only infests a few garden plants and native shrubs, whereas *C. destructor* (African white wax scale) and *C. rubens* (pink or red wax scale) are pests of citrus trees and they also attack many other introduced and native trees and shrubs. *C. rusci* (a white wax scale), similar to *C. ceriferus* though smaller, has been reported as occurring on melaleucas growing on the far north coast of New South Wales and on the Queensland coast. Honeydew is produced by the insects, often in large quantities, and sooty moulds, which develop in association, may cover the leaves, stems and fruit upon which the secretion falls.

Little is known about the composition of the honeydew secreted by coccids, and nothing appears to have been published on the honeydew produced by the genus *Ceroplastes*, although Fraser (1937) has reported briefly on some results obtained with material supplied from the early stages of this work. The coccid *Trabutina mannifera* feeding on tamarisk in Sinai produces a secretion (manna) which contains 55% sucrose, 25% invert sugar and 19·3% dextrin (Weber, 1930). Gontarski (1940) records the composition of the honeydew secreted by *Physokermes piceae* Schr. (*Lecanium hemicryphum*) as containing no fructose, 11·4% water, 28·1% dextrin, 14·3% glucose and 35·8% sucrose, and that secreted by *L. quercus* (L.) Burmeister as containing 16·3% water, 11·1% dextrin, 43·7% glucose and 39·9% sucrose. More information is available on the composition of the honeydew produced by another hemipterous family of the suborder Homoptera, namely, the family Aphididae (Michel, 1942; Molliard, 1931; Mawson, 1948). However, except for the paper by Molliard, no reference is made to the presence of sugar alcohols. Molliard reports that the excretion of *Aphis euonymi* Fabr. feeding on *Euonymus europaeus* L. contains quantities of dulcitol which are apparently obtained from the leaves of the host and pass through the insect unchanged; sugars are also present.

EXPERIMENTAL

Separation of the honeydew

From Ceroplastes destructor. A mixture of insects, wax and honeydew was collected from the branches of infested lemon trees and most of the honeydew separated by pressing and draining. The remainder of the honeydew was obtained after solution of the wax in $CHCl_3$. Material collected from the Dural district of New South Wales yielded 80·6% honeydew, 7·0% insect bodies and 12·4% $CHCl_3$-soluble material; that collected from the Gosford district of New South Wales yielded 85·8% honeydew, 1·7% insect bodies and 12·5% $CHCl_3$-soluble material. While the material was

being collected it became apparent that the amount of honeydew produced by the insects varied from tree to tree. When clarified by treatment with charcoal in the cold, a sample of the honeydew exhibited insignificant optical activity.

The honeydew produced by *C. destructor* growing on native blackthorn (*Bursaria spinosa* Cav.) was obtained by the method described below.

From Ceroplastes ceriferus *and* C. rubens. The *C. ceriferus* occurred on *Dodonaea triquetra* Wendl. (native flax) growing in the Newcastle district (New South Wales) and the *C. rubens* on the leaves of citrus trees and of Gordonia trees growing in the Gosford district, the leaves of a lillipilly (*Eugenia luehmanni* F. Muell.) growing in the Sydney district and on the leaves of a mango (*Mangifera* sp.) growing in the Brisbane district (Queensland). Because of the small amounts of material available the method described above for *C. destructor* was modified as follows:

The waxy covering was carefully removed, leaving the insect attached to the twig or leaf, and dissolved in a small volume of $CHCl_3$. The $CHCl_3$ solution was extracted with 10 ml. and 3 × 5 ml. water, the aqueous extract treated with charcoal, filtered and evaporated to dryness *in vacuo* at 40°. The residue was dissolved in 1 ml. water. By estimating the amount of $CHCl_3$-soluble material and knowing the weight of insect bodies and the weight of the waxy covering it was possible to calculate that the *C. ceriferus* material contained 44·6% honeydew, 26·3% insect bodies and 29·1% $CHCl_3$-soluble material; that of *C. rubens* growing on citrus contained 19·0% honeydew, 12·1% insect bodies and 68·9% $CHCl_3$-soluble material. As with *C. destructor*, it is to be expected that these percentages will vary, depending upon a number of factors such as the time of the year when the insect material is collected and the tree upon which the insect is feeding. However, the values do reflect the nature of the scale insects and they show that *C. destructor* and *C. ceriferus* yield more honeydew and less wax than *C. rubens*. The last insect appears to have a much harder wax than the other two and since from published information (Hackman, 1951; Kono, 1932; Koyama, 1935; Chibnall, Piper, Pollard, Williams & Sahai, 1934) the composition of the waxes are not fundamentally different, the additional hardness appears to be due to the presence of less honeydew.

Filter-paper partition chromatography

One-dimensional filter-paper partition chromatography was used for the detection of the sugars and sugar alcohols present in the honeydews. Whatman no. 1 filter-paper strips were used in both the 'descending' and the 'ascending' methods, and the experimental techniques employed were those described by Partridge (1948), Jermyn & Isherwood (1949) and Williams & Kirby (1948). As solvents the non-aqueous phases of the following systems were used (proportions are by vol.): *n*-butanol-ethanol-water (4:1:5); *n*-butanol-acetic acid-water (4:1:5) (Partridge, 1948); ethyl acetate-acetic acid-water (3:1:3); ethyl acetate-pyridine-water (5:2:5) (Jermyn & Isherwood, 1949). To detect the position of the individual sugars and sugar alcohols on the paper strips, use was made of the following four reagents: 1% (w/v) $KMnO_4$ in 2% (w/v) aqueous Na_2CO_3 (Pacsu, Mora & Kent, 1949), bromocresol purple in boric acid-borax solution (Bradfield & Flood, 1950), ammoniacal $AgNO_3$ (Partridge, 1948) and aniline hydrogen phthalate (Partridge, 1949).

Table 1. *R_F values of sugar alcohols on Whatman no. 1 paper, corrected to 20°*

Compound	Solvent systems (parts by vol.): *n*-Butanol, 4: ethanol, 1: water, 5 R_F	*n*-Butanol, 4: acetic acid, 1: water, 5 R_F	Ethyl acetate, 3: acetic acid, 1: water, 3 R_F
Inositol	0·07	0·06	0·08
Sorbitol	0·17	0·18	0·19
Dulcitol	0·17	0·16	0·18
Mannitol	0·20	0·19	0·19
Ribitol	0·28	0·25	0·28
Arabitol	0·26	0·22	—
Xylitol	0·24	0·20	—
Erythritol	0·31	0·35	0·34
Glucose*	0·185	0·18	0·17
Rhamnose*	0·345	0·37	0·34

* Published R_F values (Partridge, 1948; Jermyn & Isherwood, 1949).

Since preliminary work had shown that the honeydews produced by the wax scales contained sugar alcohols, the behaviour of a number of representative alcohols under the chromatographic conditions used was studied. In Table 1 are listed the R_F values and the solvents used. The R_F values have been corrected to 20° (cf. Partridge, 1948) and adjusted to the published values of glucose and rhamnose, which two sugars were run on the same sheets of paper as the sugar alcohols. As indicated by Bradfield & Flood (1950) and by Hough, Jones & Wadman (1950) the R_F values for sorbitol, dulcitol and mannitol are about the same as that of glucose. The detecting agent used was the $KMnO_4$-Na_2CO_3 reagent.

For each run 5 µl. of honeydew extract were used. It was found convenient to run a series of paper strips of each extract in each solvent and to detect the sugars and/or sugar alcohols present on different strips by each of the reagents mentioned. In every case a mixture of glucose and ribitol was run as a control on the same strip of paper as were the honeydew extracts. Similar results were obtained with each of the four solvents used in the tests. Only one spot was obtained on the chromatograms with the honeydew from *C. destructor* and *C. ceriferus* and this was best detected with

the $KMnO_4$ or bromocresol purple reagents. Aniline hydrogen phthalate gave a negative result, thus showing the absence of aldopentoses, aldohexoses and uronic acids. Ammoniacal $AgNO_3$ did not give uniformly satisfactory results. The sugar alcohol present in the honeydew produced by these two insects proved indistinguishable from ribitol in all four solvents when run in parallel or when mixed together. Ribitol could be readily separated from mixtures with sugars. The honeydew from *C. rubens* gave three spots, two of which were detected with aniline hydrogen phthalate. The other spot corresponded to ribitol. One of the two reducing sugars corresponded to glucose, the other had R_F 0·08 at 20° in butanol-ethanol-water. The small amount of material available and the low concentration of this component prevented its further identification. For the three insects the results just mentioned were found to be independent of the host plant.

The approximate amount of ribitol present was determined by periodate oxidation (Hough, 1950; Jackson, 1944). The ribitol was extracted from the paper with water, oxidized with sodium metaperiodate and the resultant formic acid estimated using the procedure described by Hirst & Jones (1949). It was observed that the logarithm of the amount of ribitol was very approximately proportional to the area of the spot on the filter paper. Known quantities of ribitol were used to standardize the method. The honeydew from *C. ceriferus* was found to contain 5·8%, and that from *C. rubens* on lemon trees 3·0%, ribitol. The honeydews excreted by these three species also contained protein and amino-acids as detected by colour reactions.

When comparing the ribitol content of the honeydews excreted by the three *Ceroplastes* spp. a number of factors must be taken into consideration. The concentration of ribitol in the honeydew will vary with the amount of sooty mould present which utilizes the ribitol, and will depend upon the amount of water present. The *C. ceriferus* scale examined was apparently free from sooty mould, which may account for the high ribitol content. Both the *C. destructor* and *C. rubens* specimens were thickly covered with mould growth. The fact that *C. ceriferus* and *C. destructor* do not, as far as is known, occur on the same host plant introduces another variable, and the concentration of ribitol in the sap of the host plant may well be of major importance in determining its concentration in the honeydew.

Isolation and identification of ribitol

The clarified honeydew from the *C. destructor* material collected in the Dural district (190 g.) was evaporated to dryness at 40° *in vacuo* after the addition of $CaCO_3$ (0·5 g.) to preserve a neutral reaction. The residue was extracted with hot methanol (80 ml.); from the extract flat elongated prisms were deposited. A further crop of crystals was obtained from the mother liquor on standing. Yield 5·2 g. (2·7%). (The honeydew from the *C. destructor* material collected in the Gosford district yielded 3·1% ribitol.) After crystallization from water and drying at 80°, the material had m.p. 102° (uncorr.), not lowered by admixture of an authentic sample of ribitol. (Found: C, 39·3; H, 7·9. Calc. for $C_5H_{12}O_5$: C, 39·5; H, 7·9%.)

The dibenzylidene derivative was prepared by the method of Fischer (1893). After crystallization from ethanol and from acetone containing a little light petroleum the substance melted at 173° (uncorr.). The melting point recorded by Fischer (164–165°) is lower, due probably to contamination with the monobenzylidene derivative. (Found: C, 69·3; H, 6·1. Calc. for $C_{19}H_{20}O_5$: C, 69·5; H, 6·1%.) Mixed melting point with an authentic sample of the dibenzylidene derivative of ribitol (m.p. 173°) was 173° (uncorr.).

Recognition of ribitol in plant sap of various species by paper chromatography

Twigs (without leaves; 25 g.) were cut into 1 in. lengths and extracted in a Waring Blendor with 25 ml. water. The material was filtered and the residue washed with 25 ml. water. The combined filtrate and wash water were mixed with decolorizing charcoal and filtered; $CaCO_3$ (0·5 g.) was added to the filtrate, which was evaporated to dryness at 40°. The dry residue was extracted with methanol and the methanolic solution evaporated to dryness. The residue was dissolved in water (2 ml.), mixed with charcoal and filtered. Portions of this extract (5 µl.) were used in the paper-chromatographic technique described above.

In this manner the presence of ribitol, glucose and other unidentified sugars was suggested in lemon, sweet orange and *Eugenia luehmanni* twigs. Glucose and other sugars, but no ribitol, could be detected in the twigs of *Eucalyptus globulus* (Labill.) var. *compactus*.

Ribitol in the honeydew of other scale insects

The honeydews produced by *Saissetia oleae* Bernard (black or brown olive scale) growing on the twigs of a sweet orange tree, and by *Eriococcus eucalypti* Mask. growing on *Bursaria spinosa*, were extracted and examined by the methods described for the *Ceroplastes* scales. Ribitol, glucose and other sugars were indicated in the honeydews from both insects. However, by a similar method no ribitol could be detected in the honeydew excreted by *Eriococcus coriaceus* Mask. growing on the twigs of *Eucalyptus globulus* var. *compactus*.

DISCUSSION

Apart from the work described in this paper and the earlier reference by Fraser (1937), ribitol has been reported as occurring free in only two plants, *Adonis vernalis* L. (Podwykssozki, 1889; Merck, 1893), and *Bupleurum falcatum* L. (Wessely & Wang, 1938). However, in a combined form, it is a constituent of riboflavin. Evidence has now been obtained for the occurrence of ribitol in the honeydew produced by three of the four species of wax scale insects belonging to the genus *Ceroplastes* which occur in Australia: namely, *C. destructor* growing on citrus trees and on native blackthorn (*Bursaria spinosa*); *C. ceriferus* growing on native flax (*Dodonaea triquetra*); *C. rubens* growing on citrus trees, on a mango tree (*Mangifera* sp.), on a lillipilly (*Eugenia luehmanni*) and on a gordonia tree. Fraser (1937) has noted the occurrence of ribitol in the honeydew of *C. destructor* growing on *Melia azedarach* L. var. *australasica*. For *C. destructor* and *C. ceriferus* ribitol appears to be the only simple carbohydrate material present in the honeydew.

Filter-paper partition chromatography was used to detect the ribitol. Potassium permanganate in sodium carbonate solution, and bromocresol purple in boric acid-borax solution, were found to be more satisfactory reagents for detecting sugar alcohols than ammoniacal silver nitrate as used by Hough (1950). In the case of *C. destructor* proof for the identity of the sugar alcohol was obtained by isolating the ribitol and identifying it by physical methods and by preparation of the dibenzylidene derivative.

The sap produced by three of the above-mentioned host plants has been examined by paper chromatography for the presence of ribitol, and this or a similar sugar alcohol was detected in all cases. No attempt was made to identify any other constituent of the sap. Regarding other host plants, Fernald (1903), Froggatt (1921) and Zeck (1932) list a large number of plants as hosts for these *Ceroplastes* scales, representing a large number of families distributed amongst the ferns, gymnosperms and angiosperms (both monocotyledons and dicotyledons). The only similarity between these plants is that many would be classed as 'aromatic'.

Ribitol could not be detected, by the methods used, in the honeydews produced by all coccids; e.g. not from that of *Eriococcus coriaceus* Mask. growing on eucalypts (*Eucalyptus globulus* var. *compactus*) nor was it detected in the sap of the host plant. However, ribitol was found to be present in the sap of a sweet orange tree and in the honeydew produced by *Saissetia oleae* (black or brown olive scale) growing on it, and also in the honeydew produced by *Eriococcus eucalypti* growing on *Bursaria spinosa*.

It appears highly probable that scales of the genus *Ceroplastes* ingest and excrete the ribitol unchanged. It is not known if the insect metabolizes any of the ribitol and if so what kind of ribitol balance exists. *Drosophila melanogaster* is able to utilize sugar alcohols including ribitol (Hassett, 1948). Fraser (1937) has shown that ribitol is a very satisfactory medium for the growth of the most important groups of sooty mould fungi, the Capnodiaceae and members of the Fungi Imperfecti. More satisfactory growth was made on a medium containing 2% of ribitol than on any other carbohydrate medium that was tested. It may well be that the species of sooty moulds found on plants affected by scales of the genus *Ceroplastes* depend upon their ability to utilize ribitol.

SUMMARY

1. The honeydews produced by three of the four species of wax scale insects belonging to the genus *Ceroplastes* which occur in Australia have been examined.

2. Ribitol was isolated from honeydew from *C. destructor* and identified.

3. By the use of filter-paper partition chromatography it has been indicated that the honeydews excreted by *C. destructor*, *C. ceriferus* and *C. rubens* contain ribitol. For *C. destructor* and *C. ceriferus* this appears to be the only simple type of carbohydrate material present. The honeydew excreted by *C. rubens* contains, in addition to ribitol, glucose and another (unidentified) reducing sugar.

4. The saps of three host plants for these scales were examined and found probably to contain ribitol.

5. The R_F values for a number of sugar alcohols in several solvents are given.

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The Nature and Catalytic Activities of Milk Xanthine Oxidase

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The widely accepted view that xanthine oxidase is a flavoprotein whose flavine prosthetic group is alternately reduced by the substrate and oxidized by the hydrogen acceptor has not been firmly established experimentally. It rests almost entirely on the observations of Ball (1939*a*) and Corran, Dewan, Gordon & Green (1939) that preparations of the enzyme from milk contain flavin-adenine dinucleotide (FAD). A number of investigators (Ball, 1939*a*; Corran *et al.* 1939; Horecker & Heppel, 1949; Kalckar, Kjeldgaard & Klenow, 1950) have found that the FAD in xanthine oxidase preparations is reduced when xanthine or hypoxanthine is added under anaerobic conditions, but the reduction is incomplete and, in the only study in which the rate was measured, the authors (Corran *et al.* 1939) concluded that the reduction was too slow to account for the catalytic activity of the enzyme.

Ball (1939*a*, *b*) suggested that xanthine oxidase contained a second prosthetic group in addition to FAD. He claimed to have split the enzyme reversibly into its apo-enzyme and prosthetic group moieties by prolonged dialysis. The apo-enzyme was reactivated by the supernatant obtained by heating and centrifuging an enzyme preparation but not by pure FAD. Since the supernatant solution contained FAD, Ball suggested that the active enzyme contained as prosthetic groups both FAD and an additional component present in the heated enzyme preparation. However, he pointed out that it was possible that this additional component would be active on its own. The significance of Ball's findings has been questioned by Kalckar *et al.* (1950) who found that the reactivation obtained was due to the presence in the heated supernatant of sulphydryl compounds which removed metal ions acquired by the intact enzyme during dialysis. Thus, the reversible splitting of xanthine oxidase into apo-enzyme and prosthetic group has not yet been achieved.

Corran *et al.* (1939) believed that the typical reddish tinge of the enzyme was due to a second prosthetic group. This view, however, has since been criticized by Lowry, Bessey & Crawford (1949), who have suggested that since the red tinge disappears entirely when the enzyme is denatured, it may be due to an effect of the specific protein upon the light absorption of the enzymic FAD.

More recently, Horecker & Heppel (1949) have also suggested that xanthine oxidase contains a component which is reduced by dithionite ($Na_2S_2O_4$) but not by hypoxanthine.

The present paper shows that FAD does act as a hydrogen-carrying prosthetic group of xanthine oxidase and that there is no real evidence for the existence of other prosthetic group components.

MATERIALS AND METHODS

Xanthine. A stock solution ($6{\cdot}6 \times 10^{-4}$ M) of the Roche product, free from uric acid, was prepared every week. A final concentration of $6{\cdot}6 \times 10^{-5}$ M (which Hofstee (1949) found to be optimal) was obtained by using 0·3 ml. of this solution in a final volume of 3·0 ml.

Hydrochloric acid. This reagent was adjusted by titration to the same normality as the NaOH in which the xanthine was dissolved.

Albumin. A 1% (w/v) solution of partially purified egg albumin was used.

Reduced cozymase (Co I.H_2) was prepared by reduction of 35% pure cozymase by glutamate and glutamic dehydrogenase (Slater, 1950).

Reduced coenzyme II, prepared by reduction with the stoicheiometric amount of *iso*citric acid in the presence of *iso*citric dehydrogenase, was supplied by Dr E. C. Slater.

Salicylaldehyde was freshly distilled and a suitable dilution prepared immediately before use.

Xanthopterin was a pure sample of the monohydrate kindly supplied by Prof. A. Albert.

D-*Amino-acid oxidase* was prepared according to the method of Negelein & Brömel (1939) and purified to step 1. An O_2 uptake of 157 μl./10 min. at 38° was obtained with 0·2 ml. of this preparation, 0·042 M-DL-alanine and 0·088 M-glycine buffer, pH 8·3.

Measurement of enzyme activity

The spectrophotometric method of Kalckar (1947) was used. The rate of increase of the optical density at 290 mμ. caused by the oxidation of xanthine to uric acid is measured in the spectrophotometer.

Xanthine solution (0·3 ml.), HCl (0·3 ml.) and albumin (0·1 ml.) were added to two 1 cm. silica cells. To one (the reference cell) 2·3 ml. 0·1 M-glycine or pyrophosphate buffer, pH 8·3, were added; the other (the test cell) received 2·2 ml. buffer. The enzyme was so diluted in ice-cold buffer that an addition of 0·1 ml. to the above reagents in the test cell gave a change in optical density/min, at 290 mμ.

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(ΔD_{290}/min.), of less than 0·2. Readings of optical density were made against time in the Hilger 'Uvispek' spectrophotometer. All measurements were made in duplicate. A uniform rate was found for at least 80 sec. when ΔD_{290}/min. was less than 0·2. Catalase is not required to prevent inactivation of the enzyme by H_2O_2 under these conditions. The reaction rates are proportional to the enzyme concentration when albumin is present in the concentration given above. The reaction rate is optimal at pH 8·3.

Determination of Q_{O_2}. Under the conditions used in the activity test the oxidation of 1 μg./ml. xanthine to uric acid was found to give an increase of optical density of 0·048. The concentration of xanthine oxidized per min. is thus (ΔD_{290}/min./0·048 μg.)/ml. The Q_{O_2} (μl. O_2/hr./mg. dry wt.) of the preparation $= \dfrac{(\Delta D_{290}/\text{min.}) \times 0{\cdot}147 \times 60}{0{\cdot}048 \times W}$, where 0·147 is the factor used to convert μg. xanthine to μl. O_2 (1 mole xanthine is oxidized by 1 mole oxygen) and 'W' mg. is the dry weight of the preparation used in the activity measurement.

Method of measuring the reduction of the enzyme

The effect of reducing agents on the spectrum of the enzyme was studied with the use of a glass cell (internal optical path, 5 mm.) sealed to the bottom of a Thunberg tube. The cell fitted into a special holder designed for the compartment of the Beckman Model DU spectrophotometer. Stray light was excluded from the compartment by a metal hood. The enzyme and buffer were added directly to the cell and the reducing agent to the stopper. Anaerobic conditions were achieved by alternately evacuating the tube at the water pump and flushing with N_2 freed from O_2 by passing it over heated copper. It was sufficient to flush the tube twice with N_2. The readings were made against air, the cell blank being determined separately and subsequently subtracted from the original readings.

EXPERIMENTS

Purification of xanthine oxidase from milk

The method of purification is essentially that of Horecker & Heppel's (1949) modification of Ball's (1939*a*) method.

Stage 1. The casein was precipitated by calcium phosphate from trypsin-digested buttermilk according to the procedure of Ball (1939*a*). The original volume of buttermilk was 4 l. The resultant pale-yellow, slightly opalescent, supernatant was cooled to 4° and fractionated with solid ammonium sulphate. All subsequent operations were carried out at 4–8°. The protein fraction precipitated between the limits of 30 and 65 % saturation was dissolved in 0·01 M-pyrophosphate buffer, pH 8·3, and dialysed against a large volume of the same buffer. $Q_{O_2} = 333$ at 19°; the ratio of the absorption at 280 mμ. (protein absorption) to the absorption at 450 mμ. (flavine absorption) = 17·5. Yield = approx. 35 % of the enzyme activity of the original buttermilk.

Stage 2. Stage 1 preparation was dialysed against distilled water and fractionated with solid ammonium sulphate. The precipitate appearing between 33 and 40 % saturation was collected by centrifugation and dialysed against distilled water (three changes of 2 l.) for 12 hr. The dialysed preparation was then treated with calcium phosphate gel (prepared according to Keilin & Hartree, 1951) at pH 5·6 until almost all the enzyme was adsorbed (judged by disappearance of yellow colour). The enzyme was then eluted from the gel with 0·01 M-pyrophosphate buffer, pH 8·3, until the ratio $D_{280\,m\mu}/D_{450\,m\mu}$ rose above 9·0. After dialysis against distilled water for 3 hr. the enzyme solution was fractionated by adding saturated ammonium sulphate solution (adjusted to pH 7·5 with ammonia). Most of the enzyme was collected between 46 and 53 % saturation. This fraction was dialysed against 0·01 M-pyrophosphate buffer, pH 8·3, for 6 hr. $Q_{O_2} = 694$ at 19°; the ratio $D_{280\,m\mu}/D_{450\,m\mu} = 6{\cdot}15$; percentage flavine (as FAD) = 0·435. Yield = approx. 10 % of the original activity of the buttermilk.

Reduction of xanthine oxidase

The reduction of the flavine in the xanthine oxidase preparation was studied by following the disappearance of the flavine absorption band at 450 mμ. When an excess of hypoxanthine or xanthine was added to the active enzyme preparation under the conditions described in the Methods section there was always an immediate decrease in the absorption at 450 mμ. (rapid reaction). The extent of this rapid decrease varied from one preparation to another, but in all cases studied the initial rapid decrease was followed by a much slower decrease (slow reaction). The final optical density reading was approximately the same as that obtained by the addition of sodium dithionite. With highly purified preparations of the enzyme, sodium dithionite causes a decrease of about 60 % in the total absorption at 450 mμ. (Ball, 1939*a*; Corran *et al.* 1939; Horecker & Heppel, 1949). Because it was found difficult to make accurate readings of optical density before 20–30 sec. after adding the substrate, the first reading was taken at 30 sec. The reduction of the FAD which occurred in 30 sec. is referred to as 'immediate' reduction. The percentage of the total FAD which was reduced in time t was calculated from the expression

$$\frac{D_0 - D_t}{D_0 - D_{\text{dith.}}} \times 100,$$

where D_0 is the initial optical density at 450 mμ., D_t that at time t and $D_{\text{dith.}}$ the optical density after treatment with dithionite ($Na_2S_2O_4$). Reasons are given later for assuming that the sodium dithionite-reducible optical density is due to FAD.

In Fig. 1 the percentage reduction of FAD by xanthine is plotted against time for three enzyme preparations of different activity. Curves *A* and *B* describe the reduction of the FAD of two highly active preparations which gave 72 and 50 %

'immediate' reduction respectively. Curve *C* shows a preparation with a negligible xanthine oxidase activity which shows scarcely any 'immediate' reduction. This preparation had been stored in ammonium sulphate solution (10% saturation) at approximately 5° for 8 months. When freshly prepared, 50% of the FAD was reduced 'immediately' by xanthine.

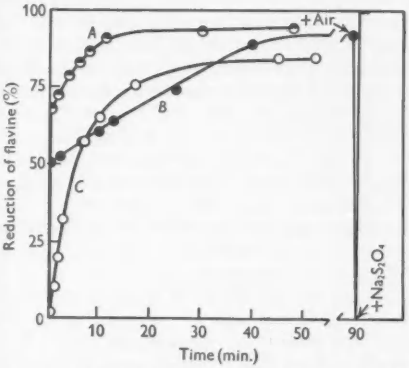

Fig. 1. The anaerobic reduction of the FAD of xanthine oxidase preparations by xanthine, showing rapid and slow reductions. Curves *A* and *B*, freshly prepared active preparations; curve *C*, preparation stored at 5° in $(NH_4)_2SO_4$ solution for 8 months. See text, p. 658 for calculation of percentage reduction.

Corran *et al.* (1939) calculated that if the function of the FAD of xanthine oxidase was that of a hydrogen carrier then, considering the activity of their enzyme preparation, the whole of the FAD should be reduced by excess hypoxanthine within 0·2 sec. of adding the substrate. The 'slow reaction' shown in Fig. 1 is very much slower than this and can contribute to only a very small fraction of the overall enzyme activity. The 'rapid reaction' might, however, be sufficiently fast to account for the enzyme activity. If this is the case, one would expect to find proportionality between the percentage 'immediate' reduction of the FAD and the activity per unit total FAD. This is tested in Fig. 2 which shows a straight-line relationship for four preparations of widely different activity. Thus that fraction of the FAD which is 'immediately' reduced can be considered to be attached to active enzyme. This enables a determination of the concentration of active enzyme in any spectroscopically clear preparation.

The secondary slow reduction of FAD observed in all xanthine oxidase preparations could be explained by any of the following hypotheses. (1) The FAD not attached to active apo-enzyme is reduced directly by xanthine in a non-enzymic reaction. (2) The FAD diffuses from inactive apo-protein to free, active apo-protein and is there reduced by xanthine. (3) FAD bound to an inactive apo-protein replaces the reduced FAD bound to active apo-protein and is there reduced by xanthine. (4) FAD which is either in the free state or is bound to inactive apo-protein is reduced by reduced FAD attached to active apo-protein.

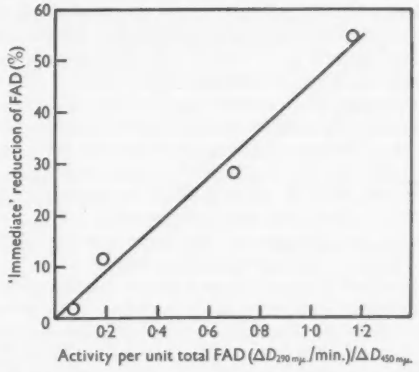

Fig. 2. Proportional relationship between the percentage of FAD 'immediately' reduced anaerobically by xanthine and the activity per unit total FAD for four xanthine oxidase preparations. Abscissa: $\Delta D_{450\,m\mu}$. is the decrease of absorption at 450 mμ. caused by addition of $Na_2S_2O_4$.

The first of these hypotheses was dismissed by direct experimentation. The FAD attached to enzyme which had been completely inactivated by treatment with cyanide (Dixon & Keilin, 1936) was not reduced when xanthine was added anaerobically. The second hypothesis was excluded by showing that when free FAD was added to the preparations there was no increase in enzyme activity. There was thus no active apo-enzyme unattached to FAD. The third hypothesis was considered unlikely because it is believed that the dissociation constant of the FAD-apo-protein complex is very small. That the fourth hypothesis is probably correct was shown by the following experiments.

In two Thunberg tubes were placed xanthine oxidase (final concentration, $0{\cdot}6 \times 10^{-5}$ M 'immediately' reducible FAD) and 1·0 ml. 0·1 M-pyrophosphate buffer, pH 8·3, and 0·1 ml. $6{\cdot}6 \times 10^{-3}$ M-xanthine added to each of the stoppers. Riboflavin (final concentration, $2{\cdot}1 \times 10^{-5}$ M) was added to one tube and pure synthetic riboflavin phosphate (final concentration, $2{\cdot}9 \times 10^{-5}$ M) to the other. After evacuation, the fluorescence of the flavines was examined visually, using a suitable blue exciting light, and the contents of the stoppers added to the tubes. The fluorescence of the riboflavin almost entirely disappeared after 15 min. and that of the monophosphate in about 25 min.

Since it is extremely unlikely that riboflavin can replace FAD on the apo-enzyme it must be assumed that, in the above experiment, the riboflavin was reduced by FAD which had itself been reduced at the apo-protein 'surface' by xanthine. A similar experiment in which the respective final concentrations of enzyme FAD (attached to active apo-protein) and added FAD were $3{\cdot}7 \times 10^{-6}$ M and $7{\cdot}7 \times 10^{-6}$ M was followed in the spectrophotometer at 450 mμ. Allowing for the known rate of reduction of the enzyme FAD it was calculated that all the added FAD was reduced in about 5 min. A further experiment of the same type in which cyanide-inactivated xanthine oxidase approximately equal in concentration to that of active enzyme was added showed that the FAD of the inactivated enzyme was reduced in about 40 min. It is, therefore, clear that the slow reductions demonstrated in Fig. 1 are due to the reduction of FAD attached to inactive apo-protein by that attached to active apo-protein.

It was of interest to determine the rate with which other flavine enzymes, in the presence of their substrates, would reduce free FAD. An active solution of notatin with glucose as the reducing substrate, at pH 5·8, required approximately 12 hr. for complete reduction of a stoicheiometric amount of free FAD. When excess glucose was added to the buffered notatin solution alone, 61 % of the total FAD was reduced in 20 sec. A secondary slow reduction, apparently complete in 2 min., of only 2 % of the FAD was thereafter recorded.

The ability of the D-amino-acid oxidase system to reduce free FAD was also investigated. A significant reduction of the FAD of this preparation by DL-alanine under anaerobic conditions could not be demonstrated. This is not surprising because the preparation was heavily contaminated with diaphorase and other pigments. However, in the presence of a small concentration of the enzyme and its substrate the reduction of added free FAD would have been detected if the reduction had occurred. No reduction of the free FAD could be demonstrated in 90 min. This agrees with the findings of Negelein & Brömel (1939) who found that the apo-enzyme of D-amino-acid oxidase, when saturated with FAD and in the presence of D-alanine under anaerobic conditions, did not appreciably reduce added FAD.

It was also of interest to find that xanthine, in the presence of xanthine oxidase, was able to reduce the FAD of notatin. This experiment was carried out similarly to those just described. Suitable concentrations of notatin and xanthine oxidase were placed in the optical cell fused to the Thunberg tube and xanthine solution added anaerobically. The reduction of FAD as measured by the decrease in absorption proceeded past the theoretical value for complete reduction of the FAD of the xanthine oxidase preparation, and it became apparent that there was a steady but slow reduction of the FAD of the notatin.

The linkage between FAD and the apo-enzyme of xanthine oxidase

During a study of possible procedures for the purification of xanthine oxidase, it was found that saturation of the enzyme preparation with magnesium sulphate, followed by dialysis to remove the salt, greatly decreased the activity of the enzyme and altered its spectrum in the visible region. The main alteration of spectrum lay in the partial removal of the flavine peak at 450 mμ. It was also noticed that when the enzyme preparation was saturated with magnesium sulphate and allowed to stand for 30 min. at 10°, the preparation lost some of its reddish tinge and was yellower. Examination of the dialysis water under ultraviolet light showed a strong yellow fluorescence.

A study of this effect of magnesium sulphate revealed that this salt and some other bivalent metal salts were able to detach the FAD from the enzyme protein. This removal could be followed in the spectrophotometer by the decrease in absorption at 450 mμ. In high concentration, magnesium sulphate or calcium chloride do not affect the absorption spectrum of free FAD. By saturating an enzyme preparation with calcium chloride most of the FAD was detached in 30 min. at room temperature or in a few minutes at 38°. When this reaction was carried out at room temperature, 75 % or more of the FAD could be detached without precipitating any of the protein. The whole of the enzyme FAD could be detached without protein precipitation if the reaction was carried out at 0°. The preparation was bright yellow after this treatment and had lost all traces of the reddish tinge associated with the intact enzyme. It was also found that merely by holding the salt-free preparation at 38° some FAD was detached before any protein was precipitated. Not only could the FAD detached from the apo-protein in the above experiments be dialysed from the preparations, but the increase in fluorescence due to the increased concentration of the free FAD was most marked. When bound in the intact enzyme the FAD of xanthine oxidase is not fluorescent (see p. 662).

Treatment of the enzyme preparation with divalent metal salts such as calcium chloride thus provided a convenient means of studying the effect of the FAD-protein linkage on the spectrum of the enzyme FAD without the danger of adsorption of some of the liberated FAD on precipitated protein. In Fig. 3 the spectra of the enzyme plotted before and after treatment with calcium chloride are given together with the difference spectrum.

The difference spectrum is obviously not that of FAD. The spectrum of FAD has maxima at 375 and at 450 mμ. whereas the above difference spectrum shows no maximum at 375 mμ. and a maximum in the green at 480 mμ. The only known reaction occurring when the enzyme is treated with calcium chloride is the breaking of the protein-FAD linkage. This suggests that the difference spectrum in Fig. 3 is partly or wholly derived from absorption which is due to an effect of this linkage upon the absorption of the enzyme FAD. Thus it seems clear that the combination of FAD with the enzyme leads to considerable spectral changes.

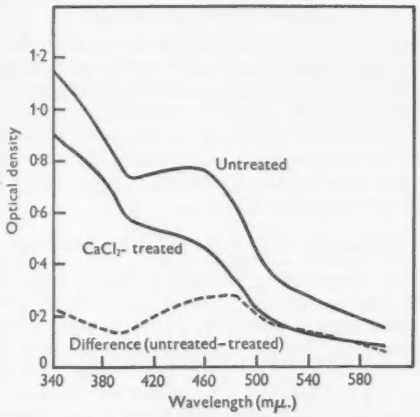

Fig. 3. The effect of $CaCl_2$ on the spectrum of xanthine oxidase. $CaCl_2$-treated, 2·9 ml. active enzyme solution +0·4 ml. saturated $CaCl_2$, incubated at 15° for 90 min. Untreated, 2·9 ml. same enzyme solution + 0·4 ml. water.

In Fig. 4 the linearity of the relationship between decreasing absorption at 520, 480 and 450 mμ. on the one hand, and decreasing activity of the enzyme on the other, is demonstrated for an enzyme preparation treated with calcium chloride. If it can be assumed that at zero activity of the enzyme calcium chloride has no further effect upon the spectrum of the preparation we can, by extrapolating to zero activity at each wavelength, plot a portion of the spectrum of the calcium chloride-treated preparation. This agrees closely with that shown in Fig. 3. Thus the curve for calcium chloride-treated enzyme in Fig. 3 corresponds to complete inactivation.

Fig. 5 shows the spectrum of intact xanthine oxidase before and after complete reduction with xanthine. Above 400 mμ., reduction with sodium dithionite gives a similar curve (sodium dithionite absorbs below 400 mμ.). The effect of reduction on the spectrum of the enzyme preparation is shown by the difference curve. The fraction which is rapidly reduced shows the same type of difference spectrum as that which is slowly reduced (Fig. 6). Also shown in Fig. 5 is the difference spectrum (oxidized –

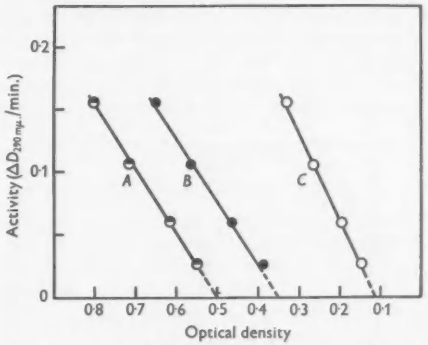

Fig. 4. The linear relationship between the activity of a xanthine oxidase preparation and the decrease in absorption at 450 (curve *A*), 480 (curve *B*) and 520 mμ. (curve *C*) caused by treatment with $CaCl_2$ (12% saturation). The $CaCl_2$-enzyme mixture was incubated in the spectrophotometer at room temperature. Samples (0·1 ml.) were removed at appropriate intervals and, after dilution in 0·4 ml. 0·1 M-glycine buffer, pH 8·3, the activity measured.

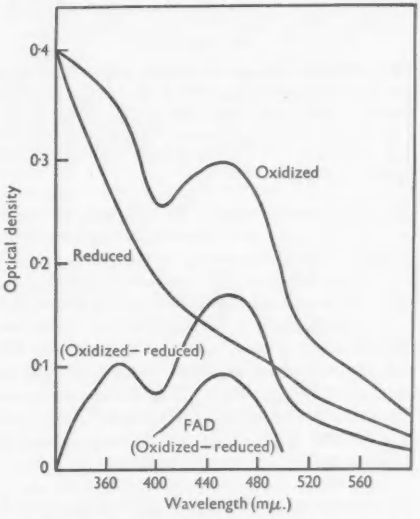

Fig. 5. The spectra of oxidized and reduced (with xanthine) xanthine oxidase plus the difference spectrum (oxidized – reduced). The difference spectrum (oxidized – reduced) of FAD equal in concentration to that in the enzyme preparation is shown for comparison.

reduced with sodium dithionite) for that concentration of FAD present in the enzyme preparation. The amount of FAD was determined by two methods:

(1) spectrophotometric determination of the optical density at 450 mμ. of the supernatant obtained by heating and centrifuging the enzyme; (2) enzymic determination of FAD on this same solution, using the D-amino-acid oxidase apo-enzyme of Negelein & Brömel (1939). The two methods gave figures within 12% of one another, thus confirming Horecker & Heppel (1949) and Lowry *et al.* (1949).

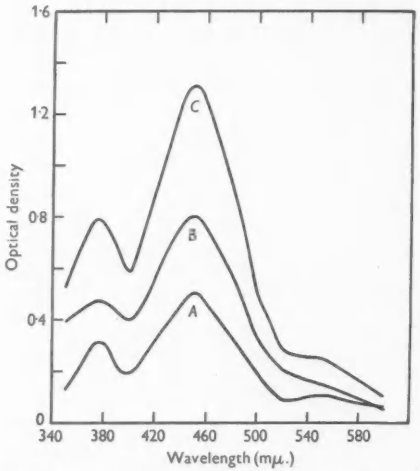

Fig. 6. Difference spectra (oxidized – reduced) obtained during the anaerobic reduction of the FAD of xanthine oxidase preparation by xanthine. (*A*) 58–100% reduction of FAD; (*B*) 0–58% reduction; (*C*) 0–100% reduction. 46% of the FAD of the preparation was anaerobically reduced by xanthine in 30 sec.

The difference spectrum for the enzyme differs from that of FAD in two important respects: (1) the intensity of the absorption is considerably greater, e.g. the absorption at 450 mμ. is 1·71 times that of the same concentration of FAD; (2) the shape of the curve is significantly different, the enzyme showing relatively much greater absorption than free FAD above 480 mμ. (cf. Ball, 1939*a*). Thus, Fig. 5 confirms the conclusion drawn from the measurements with calcium chloride-treated enzyme that combination of the FAD with the apo-enzyme causes changes in the spectrum of the former.

The non-reducible absorption of xanthine oxidase preparations in the visible spectral region

The enzyme preparation shows considerable absorption after reduction (Fig. 5). This 'residual' absorption has no sharp bands, but increases steeply towards the ultraviolet. It has been noted by previous workers (Ball, 1939*a*; Horecker & Heppel, 1949), who found that it largely disappeared when the enzyme was heated. This has been confirmed in the present study by the observation that the enzymic and spectrophotometric methods of determining FAD in the heated enzyme were in good agreement. Fig. 3 suggests that this residual absorption is not appreciably affected by calcium chloride treatment, but further work is necessary to clarify this point. It seems possible that the substance responsible for the residual absorption might be removed by precipitation at the same time as the proteins are denatured by heat. It remains to be seen whether the 'residual' absorption is a property of the enzyme or is due to an impurity.

Inhibition of D-*amino-acid oxidase and notatin by calcium chloride*

Calcium chloride was also found to inactivate the flavine enzymes, notatin and D-amino-acid oxidase, presumably by splitting off the FAD. Treatment of active solutions of these enzymes (buffered in glycine buffer) with equal volumes of saturated calcium chloride for 45 min. at 22° inactivated the notatin and D-amino-acid oxidase solutions by 73 and 89% respectively.

Fluorescence of xanthine oxidase preparations

All preparations of xanthine oxidase examined in these studies exhibited a distinct fluorescence when irradiated with blue light. Examination of these preparations by Dr G. Weber showed that the fluorescence was completely unpolarized (p is less than 0·01). Thus the fluorescence was emitted by molecules having relaxation times of the rotation much shorter than 10^{-8} sec. (the lifetime of the excited state of the fluorescence of riboflavin and FAD; Weber, 1950). Moreover, the appearance of polarized fluorescence when 1 vol. of the enzyme preparation was added to 3 vol. of glycerol showed that the absence of polarization was due to rapid rotation. It seems certain, therefore, that FAD bound to protein was not concerned in the emission of fluorescence from the enzyme preparation.

The relationship between xanthine, aldehyde and pterin oxidases

The experiments described earlier in which the anaerobic reduction of xanthine oxidase FAD was followed spectrophotometrically have been repeated using salicylaldehyde and xanthopterin as well as xanthine.

An active enzyme preparation ($Q_{O_2} = 333$ at 19°) was dissolved in 0·2 M-phosphate buffer, pH 6·8. Concentrations of xanthine and salicylaldehyde approximately five times the molar concentration of enzyme FAD and 0·1 ml. of a saturated solution of xanthopterin (in 0·01 N-sodium hydroxide) were added anaerobically in separate Thunberg tubes to the buffered enzyme solution. The reduction of the

enzyme FAD at 450 mμ. was followed as described earlier in this paper.

The reductions obtained within 30 sec. of adding xanthine, salicylaldehyde or xanthopterin were 35·8, 34·7 and 32·6 % respectively of the total FAD; eventually 97·5, 98·2 and 94·4 % respectively of the total FAD was reduced. Further experiments were made in which mixtures of the same amounts of the above substrates were added anaerobically to a similar sample of the enzyme. A mixture of salicylaldehyde and xanthine reduced 33·3 % of the total FAD in 30 sec., proceeding to a final reduction of 100·6 %, and a mixture of salicylaldehyde and xanthopterin gave corresponding reductions of 36·7 and 97·4 %.

The fact that all three substrates, both independently and in mixtures, reduced the enzyme flavine prosthetic group to the same extent indicates that the same flavine molecules take part as hydrogen carriers in the oxidation of these substrates.

The relationship between xanthine oxidase and milk diaphorase

An experiment similar to that described above was made with reduced cozymase as substrate. The molar concentration of Co I.H_2 used was approximately 16 times that of the enzyme FAD. In Table 1 the rate and extent of reduction by Co I.H_2 and by xanthine are compared.

Table 1. *Comparison of the reduction of FAD of xanthine oxidase by reduced cozymase and by xanthine*

Time after adding substrate (min.)	Reduction of enzyme FAD at 450 mμ. (%)	
	Co I.H_2	Xanthine
0·4	1	43·3
3·0	8·3	48·3
7·0	20·0	53·3
13·0	33·3	65·0
22·0	45·0	76·7
30·0	60·0	88·4
69·0	60·0	93·4

The slow reduction of the enzyme FAD by Co I.H_2 solutions, demonstrated in Table 1, was repeated with several Co I.H_2 preparations. Reduced coenzyme II (Co II.H_2), also considerably in excess of the enzyme FAD, reduced the enzyme flavine under anaerobic conditions even more slowly than Co I.H_2. The oxidation of Co II.H_2 by the enzyme preparation, in the presence of methylene blue, was negligible.

The slow reduction of xanthine oxidase flavine by Co I.H_2 may be explained in two ways: (1) the FAD attached to a very small concentration of diaphorase in the preparation was reduced by Co I.H_2 and in turn slowly reduced the FAD of xanthine oxidase, (2) by direct reduction of the FAD by Co I.H_2. Singer & Kearney (1950) have demonstrated that free flavines are reduced by Co I.H_2 and it is therefore likely that the bound FAD may be slowly reduced. There is no doubt, however, that the diaphorase activity of milk preparations is enzymic.

DISCUSSION

Reduction of xanthine oxidase

When hypoxanthine or xanthine is added to a solution of active xanthine oxidase under anaerobic conditions the reactions may be written as in equations (1) and (2) respectively:

$$\text{hypoxanthine} + \text{oxidized enzyme} \rightleftharpoons \text{xanthine} + \text{reduced enzyme}, \quad (1)$$

$$\text{xanthine} + \text{oxidized enzyme} \rightleftharpoons \text{uric acid} + \text{reduced enzyme}. \quad (2)$$

The potentials of the hypoxanthine-xanthine and xanthine-uric acid systems have been given as $E'_0 = -407$ and -355 mV. respectively at pH 7·0, whereas that of the flavoprotein system is -80 mV., or higher (Kalckar, 1941). Thus, when the purines are in excess over the enzyme, the equilibria in the above reactions would be expected to be greatly in favour of the reduction of the enzyme flavine.

Experiments described in this paper have shown that the FAD of active xanthine oxidase is very rapidly reduced anaerobically by substrates of the enzyme. Together with the finding that the activity per unit total FAD for several preparations is proportional to the percentage of rapidly reducible FAD this is strong experimental evidence, hitherto lacking, that the FAD of xanthine oxidase does indeed act as a hydrogen carrier.

The rapid reduction of part of the FAD in the preparation is followed by a slow reduction of the remainder. This slow reduction has been shown to be due to the reduction of FAD attached to inactive protein by FAD attached to active apo-protein. It might be thought that the inactive flavoprotein was merely a contaminant of the preparations. However, the following evidence indicates that it is probably inactivated xanthine oxidase. (1) The enzyme preparations were highly purified. (2) Fresh preparations in which 72 % of the total FAD was reduced within 30 sec. of adding xanthine anaerobically showed, after ageing or other treatment, relatively little 'immediate' reduction and a correspondingly greater slow reduction. (3) The difference absorption spectrum (oxidized – reduced) of the slowly reduced flavine was very similar to that of the rapidly reduced flavine. This difference spectrum is not that of a typical flavoprotein which does not absorb so far into the longer wavelengths.

Previous reports (Corran *et al.* 1939; Horecker & Heppel, 1949) of the slow or incomplete reduction of the enzyme at 450 mμ. were thus due to the presence of inactivated enzyme in their preparations. It was found, in opposition to Horecker & Heppel (1949) but in agreement with Ball (1939*a*), that eventually hypoxanthine decreased the absorption of the preparation at 450 mμ. to the same extent as sodium dithionite.

The finding that a considerable percentage of the FAD of carefully prepared xanthine oxidase preparations is attached to inactive enzyme protein explains the experiments of Lowry *et al.* (1949) on the inhibition of pterin oxidase (which is identical with xanthine oxidase; see p. 662) by 2-amino-6-formyl-4-hydroxypteridine which is a remarkably powerful competitive inhibitor of the enzyme. Lowry *et al.* found that the dissociation constant of the enzyme-inhibitor complex is so small that it 'permits a virtual titration of the enzyme'. A study of the results of kinetic experiments using the above inhibitor suggested to Lowry *et al.* that only 60% of the FAD in their preparation was associated with active enzyme centres. The alternative explanation given by Lowry *et al.*, namely that 2 moles of flavin coenzyme were associated with each active centre, is made unnecessary by the present work.

Turnover number

Since the percentage of rapidly reducible FAD in any preparation is known, it is possible to calculate the concentration of FAD combined with active enzyme present from the total FAD concentration. This has enabled the turnover number of the enzyme to be computed more accurately than hitherto. For the enzyme purified to stage 1 (see Methods) the concentration of active enzyme in the activity test (see Methods) was found to be

$$\frac{1{\cdot}68 \times 10^{-5} \times 0{\cdot}383}{120} = 5{\cdot}37 \times 10^{-8}\text{ M},$$

where $1{\cdot}68 \times 10^{-5}$ is the concentration of total FAD in the undiluted enzyme, 120 is the dilution factor in the activity test and the factor 0·383 is introduced because only 38·3% of the FAD of the preparation was 'immediately' reducible anaerobically by xanthine.

At 19° and under optimal conditions of pH (8·3) and substrate concentration ($6{\cdot}6 \times 10^{-5}$ M), ΔD_{290}/min. in the activity test was 0·129. Since the oxidation of 1 μg./ml. xanthine to uric acid was found to give an increase of optical density of 0·048, the concentration of the xanthine oxidized per minute by the stage 1 enzyme was

$$\frac{0{\cdot}129}{0{\cdot}048 \times 152{\cdot}1 \times 10^{3}} = 1{\cdot}765 \times 10^{-5}\text{ M},$$

where 152·1 is the molecular weight of xanthine. Thus the turnover number, which is defined as the number of molecules of substrate oxidized/molecule of enzyme/min., equals

$$\frac{1{\cdot}765 \times 10^{-5}}{5{\cdot}37 \times 10^{-8}} = 329\text{ min.}^{-1}$$

(at 19°). The turnover number for the stage 2 enzyme preparation was found to be 298 min.$^{-1}$ so that the average turnover number = 313 min.$^{-1}$ at 19°. Assuming the Q_{10} for the enzyme to be 1·5 (Lowry *et al.* using 2-amino-4-hydroxypteridine as substrate) the turnover number at 38° would be $313 \times 2{\cdot}11 = 660$.

Does FAD act as a carrier between different flavoproteins?

It was shown earlier in this paper that xanthine oxidase, in the presence of xanthine and under anaerobic conditions, required approximately 5 min. to reduce completely a concentration of free FAD double that of the enzyme FAD. The D-amino-acid oxidase and notatin systems required very much longer time. The experiment with the xanthine oxidase system shows that the free FAD would have a turnover number of only 0·4 min.$^{-1}$ when acting as a hydrogen carrier linking this system with other flavine dehydrogenase systems. Such a slow reaction must be insignificant physiologically. It therefore seems that free FAD is not able to act as a carrier linking any of the above systems with other flavine dehydrogenase systems in the same way that coenzyme I can, *in vitro*, link the triosephosphate and lactic dehydrogenases in the reconstructed glycolytic cycle.

Substrate specificity

The finding that the same molecules of FAD which act as the hydrogen-transferring prosthetic group in xanthine oxidase also have the same function in the oxidation of salicylaldehyde and xanthopterin lends strong support to the belief that one and the same enzyme is concerned in the oxidation of the three compounds. There is already considerable evidence in the literature that the protein 'active centres' of xanthine, aldehyde and pterin oxidases are identical (see review by Dixon (1938) and papers by Lowry *et al.* (1949), Hofstee (1949) and Krebs & Norris (1949*a*, *b*).

It is significant that Co I.H_2, in a molar concentration of 16 times that of enzyme FAD, failed to reduce the enzyme FAD significantly within 1 min. Lowry *et al.* found that reduced coenzyme I was oxidized by a xanthine oxidase preparation with a velocity only 3 or 4% that of xanthine. These authors calculated the turnover number of their enzyme with xanthine as substrate to be about 210. 3% of this value would give a turnover number of

their enzyme with Co I. H_2 as substrate of 6·3, that is the enzyme FAD would be reduced, under anaerobic conditions, in approximately 10 sec. As the diaphorase activity of the preparation used in the present experiments was at least as great as that used by Lowry *et al.* these experiments must be considered as definite evidence that the FAD molecules acting as hydrogen carriers for the xanthine oxidase activity of the enzyme do not also take part in the oxidation of Co I. H_2.

Corran *et al.* (1939) were the first to show that the xanthine oxidase activity of milk preparations could be destroyed without affecting their diaphorase activity. Moreover, Lowry *et al.* (1949) have shown that 2-amino-6-formyl-4-hydroxypteridine, which is a powerful competitive inhibitor of xanthine, aldehyde and pterin oxidase, does not inhibit milk diaphorase. Since both prosthetic groups and protein 'active centres' for xanthine oxidase and milk diaphorase are different we must now regard these enzymes as separable entities.

Prosthetic group of xanthine oxidase

The red colour of xanthine oxidase solutions has been shown to be due to the effect of the FAD-protein linkage upon the spectrum of the enzyme FAD and not to the presence of a second, coloured, component of the prosthetic group of the enzyme. Little is known about the protein-FAD linkage and the cause of its rupture by calcium chloride.

Ball (1946), in an examination of the yellow flavoprotein prepared from yeast by Kunitz & McDonald (1946), found that it had an absorption spectrum in the visible region similar to that of xanthine oxidase except that the absorption at wavelengths greater than 500 mμ. was virtually absent. An acidified chloroform extraction of this yellow flavoprotein, after denaturation, produced a solution having absorption maxima in acid solution at 375, 285 and 265 mμ. Ball considers that this almost colourless substance may also be a component of other flavoprotein enzymes which have similar atypical flavoprotein absorption spectra and that these enzymes contain two or more prosthetic groups. These enzymes were stated to be: (1) the yellow flavoprotein of Kunitz & McDonald (1946); (2) the yeast flavoprotein described by Green, Knox & Stumpf (1941); (3) xanthine oxidase; (4) liver aldehyde oxidase; and (5) glucose oxidase. It should be pointed out that neither of these yeast flavoproteins has yet been found to possess catalytic activity. Extraction of the supernatant from a heat-denatured xanthine oxidase preparation of high purity in the present studies did not yield a compound with absorption maxima as given by Ball. The only extractable material showed, in aqueous solution of pH 5·5, a general absorption without maxima in the visible region of the spectrum. In view of: (*a*) the disappearance of the absorption not reducible by xanthine when the enzyme is denatured by heat; (*b*) the complete lack of evidence to show that a substance giving this absorption has a catalytic function (e.g. it is not reduced by substrates of the enzyme) and (*c*) the fact that FAD does act as a hydrogen carrier as shown by its 'immediate' reduction by xanthine, there seems no real evidence to suggest that xanthine oxidase contains any such second prosthetic group.

SUMMARY

1. Xanthine or hypoxanthine anaerobically reduce the flavin-adenine dinucleotide (FAD) of all xanthine oxidase preparations studied in two ways: (*a*) a rapid reduction, occurring within 30 sec.; (*b*) a slow reduction which may take up to 60 min. or more.

2. The activity per unit total FAD of four enzyme preparations is proportional to the percentage of FAD which is rapidly reducible by xanthine. This confirms the generally held belief that the FAD of active xanthine oxidase acts as a hydrogen carrier.

3. The slow reaction is due to the reduction of FAD attached to inactivated apo-enzyme by reduced FAD attached to active apo-enzyme.

4. Xanthine oxidase, D-amino-acid oxidase and notatin, in the presence of their substrates, anaerobically reduce free FAD so slowly that free FAD cannot act significantly *in vivo* as a carrier linking these systems with other flavine dehydrogenases.

5. Calcium chloride, magnesium sulphate and some other bivalent metal salts are able to detach the FAD of xanthine oxidase from the apo-protein without precipitating the latter. Spectrophotometric analysis of this reaction shows that the linkage between FAD and the apo-enzyme increases the absorption due to FAD by a factor of 1·71 at 450 mμ. It is also responsible for the reddish colour of xanthine oxidase preparations. There is no evidence for an additional coloured component of the prosthetic group of this enzyme, first postulated by Corran *et al.* (1939).

6. The same FAD molecules in a xanthine oxidase preparation act as hydrogen carriers in the oxidation of xanthine or hypoxanthine, salicylaldehyde and xanthopterin. This is further very strong evidence that the enzymes xanthine oxidase, aldehyde oxidase and pterin oxidase, occurring in milk, are one and the same enzyme.

7. The same molecules in xanthine oxidase preparation do not act as hydrogen carriers in the oxidation of xanthine and reduced cozymase. Since other workers have shown that the active centres for these reactions are different these enzymes must now be regarded as separate entities.

8. The minimum value for the turnover number of xanthine oxidase is 313 min.$^{-1}$ at 19°.

9. Xanthine oxidase has virtually no fluorescence when irradiated with blue light, compared to free FAD.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in this work and Dr E. C. Slater for helpful discussions. I also wish to thank the Australian National University for a scholarship.

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The Reduction of Cytochrome *c* by Hypoxanthine and Xanthine Oxidase

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The work by Bigwood, Thomas & Wolfers (1935) and Horecker & Heppel (1949) showing that xanthine oxidase, in the presence of its substrate, is able to reduce oxidized cytochrome *c* is of interest since it suggests the possibility that the reduced flavin prosthetic group of the enzyme may be re-oxidized via a cytochrome system rather than directly by oxygen. A puzzling finding by Horecker & Heppel was that the reduction of cytochrome *c* was very slow anaerobically and that the rate of the reduction increased markedly with increasing oxygen tension.

The present paper deals with a further study of the reduction of cytochrome *c* by hypoxanthine in the presence of xanthine oxidase. The reduction of cytochrome *c* under both anaerobic and aerobic conditions has been confirmed, but no evidence that oxygen increases the rate of this reduction has been found.

METHODS

The reduction of cytochrome *c* was followed at 550 mμ. in the Beckman spectrophotometer using a slit width of 0·1 mm. For anaerobic experiments a Thunberg tube fused to a 5 mm. optical glass cell contained the reaction mixture (see below). The reaction was started by adding the hypoxanthine from the hollow stopper. The readings were made against air. Anaerobic conditions were achieved by exhausting the tube at the pump and by flushing twice with N_2 freed of O_2.

Aerobic experiments were made in open 1 cm. cells. The readings were made against a reference cell containing water. In plotting the results of anaerobic experiments the cell 'blank' (determined separately) was subtracted from the optical density reading and the resultant figure multiplied by two. This facilitated comparison between data for aerobic and anaerobic experiments.

Unless otherwise stated, the reaction mixture, in both aerobic and anaerobic experiments, contained $1{\cdot}45 \times 10^{-5}$ M-cytochrome *c*, $3{\cdot}2 \times 10^{-4}$ M-hypoxanthine, 0·38 mg./ml. albumin, 0·05 ml. of catalase and 0·1 ml. xanthine oxidase preparation in a final volume of 3·2 ml.

Cytochrome c. Preparations containing 0·34% Fe, prepared by the method of Keilin & Hartree (1945), were kindly supplied by Dr C. L. Tsou. The preparations were thoroughly dialysed against distilled water.

Albumin. A highly purified horse serum albumin was kindly supplied by Dr E. F. Hartree.

Catalase. This preparation was made according to the method of Keilin & Hartree (1937), omitting the ultrafiltration step; it contained some ferritin. It was thoroughly dialysed against distilled water.

Hypoxanthine solutions. ($7{\cdot}35 \times 10^{-4}$ M). These solutions were prepared in distilled water every 3 days from the pure Roche product and kept at 5° when not in use.

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Xanthine oxidase. The enzyme was prepared from milk as described in a previous publication (Morell, 1952). The preparations were dialysed against distilled water and diluted at 0° before use so that an optical density change at 550 mμ. of approximately 0·02/min. was obtained in the measurement with oxidized cytochrome *c*.

The concentrations of cytochrome *c*, albumin and hypoxanthine were the same as those used by Horecker & Heppel (1949). A very much higher concentration of catalase was used in the present study to obtain consistent results under the present conditions. In the aerobic experiments it was desired completely to break down the H_2O_2 formed by the oxidation of hypoxanthine. Dixon (1925) has shown that relatively large concentrations of catalase are necessary for this purpose. Horecker & Heppel (1949) used phosphate buffer, pH 7·4 (final concentration not stated), in their reaction mixture. In the present study, unless stated otherwise, the reaction mixtures did not contain added buffers because it was found that even low concentrations of buffer solution inhibited the reduction (see p. 668). The pH of the above reaction mixture was found to be 6·8 when measured with the glass electrode.

EXPERIMENTS

Comparison of the anaerobic and aerobic rates of reduction

In Fig. 1, curves *A* and *B* represent the changes in optical density at 550 mμ. with time, for the anaerobic and aerobic reactions respectively. Even anaerobically the cytochrome *c* was not fully reduced by hypoxanthine. This can be seen in Fig. 1 by comparing the extent of reduction caused by hypoxanthine (curve *A*) with that caused by the subsequent addition of dithionite (curve *A*, $+Na_2S_2O_4$). In fact only about 84 % reduction was achieved anaerobically and 52 % aerobically. These results were reproducible with the same enzyme preparation although, with different preparations, differences in the degree of reduction under aerobic conditions were found. The degree of reduction achieved could not be correlated with the purity of the enzyme preparations.

It can also be seen in Fig. 1 that, whereas the anaerobic reduction rate is almost linear for most of the reaction, the reduction under aerobic conditions is approximately logarithmic. Many enzyme and cytochrome *c* preparations have been examined in these studies and in all cases the anaerobic reduction rate was found to be greater than the aerobic reduction rate. Even the use of very high concentrations of catalase (0·2 ml. strong solution in 3·2 ml. mixture) did not affect the shape of the aerobic reduction. The shape of this curve, in the presence of a high concentration of catalase, is probably largely due to the fact that oxygen is competing with cytochrome *c* as the hydrogen acceptor.

The admission of air in the anaerobic experiment when the reduction was at a maximum (Fig. 1; +air) caused a rapid re-oxidation of the reduced cytochrome. Other experiments showed that partial re-oxidation occurs even in the presence of catalase. The cytochrome was almost completely re-oxidized in the absence of catalase. The complete reduction of the cytochrome *c* by dithionite after it had been oxidized in the absence of catalase (see Fig. 1) showed that the hydrogen peroxide was, in fact, oxidizing the reduced cytochrome and not destroying it.

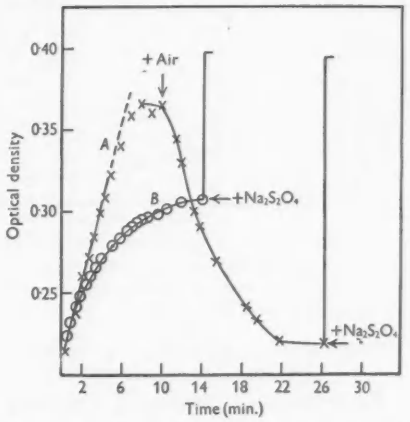

Fig. 1. The reduction of oxidized cytochrome *c* (purified) by hypoxanthine and xanthine oxidase; curve *A*, anaerobic conditions; curve *B*, aerobic conditions. The reaction mixture was as described under Methods except that catalase was omitted for the anaerobic experiment. Separate experiments showed that catalase did not affect the anaerobic reduction.

The rate of reduction of cytochrome *c* by xanthine oxidase and hypoxanthine was found to be proportional to the concentration of the enzyme. The reduction did not occur in the absence of either the enzyme or its substrate. These results confirm those of Horecker & Heppel (1949).

With either xanthopterin or salicylaldehyde as substrate of the enzyme, the cytochrome *c* was reduced under anaerobic conditions. With xanthopterin as substrate, however, the reduction was very slow. Aerobically the reduction did not proceed at a measurable rate with salicylaldehyde as substrate. Aldehydes are known to inactivate xanthine oxidase under aerobic conditions (Horecker & Heppel, 1949; Corran, Dewan, Gordon & Green, 1939).

In Fig. 2 the inhibitory effect of various concentrations of phosphate buffer on the rate of anaerobic reduction of cytochrome *c* by hypoxanthine and xanthine oxidase is shown. Even the addition of phosphate buffer, pH 7·3, to a final concentration of $3·2 \times 10^{-3}$ M caused a 44 % inhibition. Glyoxaline

and glycine buffers of the same pH and concentration similarly inhibited the reduction. Sodium and potassium chloride also effectively inhibited the reduction. This inhibition by buffers and salts seems to be due to a general salt effect and not to the toxicity of a particular ion.

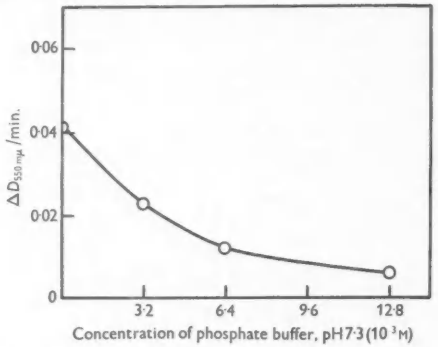

Fig. 2. The effect of phosphate buffer, pH 7·3, in inhibiting the anaerobic reduction of oxidized cytochrome *c* by hypoxanthine and xanthine oxidase.

The reduction of 'endogenous' cytochrome c of heart-muscle preparation

Keilin & Hartree (1949) believe that the cytochrome *c* present in a colloidal heart-muscle preparation is organized with respect to cytochrome oxidase and other components of the succinoxidase system in a manner similar to that existing in the cell. It was of interest in the present experiments, therefore, to compare the ability of the xanthine oxidase system to reduce this 'endogenous' cytochrome *c* with its ability to reduce the purified, extracted pigment. For this experiment heart-muscle preparation, prepared according to the method of Keilin & Hartree (1947) by Dr C. L. Tsou, was washed several times in distilled water, after precipitation at pH 5·5, to remove salts which inhibit the reduction, and finally suspended in distilled water.

When xanthine oxidase was added to the heart-muscle preparation plus hypoxanthine under anaerobic conditions and viewed under a low dispersion microspectroscope the *a*- and *c*-bands of cytochrome at 604 mμ. and 550 mμ. appeared slowly. For the comparison of the rates of reduction of 'endogenous' and soluble cytochrome *c*, an additional Thunberg tube was used with the same reagents as described above, except that the heart-muscle preparation was replaced by an approximately equal concentration of soluble cytochrome *c* (determined by comparing the intensities of fully reduced 'endogenous' and soluble cytochrome *c*).

On adding the enzyme in the stoppers simultaneously to the cytochrome-hypoxanthine mixtures and on comparing the increase in the intensities of the α-band of cytochrome *c*, it was found that the rate of reduction of 'endogenous' cytochrome *c* was only about one-quarter that of soluble cytochrome *c*. Although it must be admitted that the method of comparing these rates is not very accurate it is certain that, under these conditions, the 'endogenous' cytochrome *c* is reduced at a slower rate than the soluble form.

DISCUSSION

The present study does not confirm Horecker & Heppel's (1949) finding that oxygen stimulates the reduction of cytochrome *c* by xanthine oxidase and hypoxanthine. Fig. 1 shows that oxygen has the inhibitory action expected from its ability to compete with cytochrome *c* as the hydrogen acceptor. In agreement with this finding Singer & Kearney (1950) have shown that the rate of reduction of cytochrome *c* by leucoriboflavin is decreased by oxygen.

Since milk xanthine oxidase reacts rapidly with oxygen, it is usually considered that this enzyme belongs to a small group of dehydrogenases (the aerobic dehydrogenases) which react directly with oxygen without the intervention of intermediary hydrogen carriers. The finding by Bigwood *et al.* (1935) and Horecker & Heppel (1949), confirmed in the present paper, that xanthine oxidase reacts with cytochrome *c* raises the question whether oxygen is the immediate hydrogen acceptor *in vivo*. The fact that the initial rate of reduction of cytochrome *c* is the same in the presence as in the absence of oxygen (Fig. 1) shows that the rate of reaction of the enzyme with cytochrome *c* is comparable with the rate with 20% oxygen. The concentration of cytochrome *c in vivo* is not known, but the oxygen tension is considerably less than 20%, and Horecker & Heppel (1949) have shown that the rate of the aerobic reaction decreases markedly with decreasing oxygen tension. It was found in the present study that xanthine oxidase reacted considerably more slowly with the 'endogenous' cytochrome *c* of a heart-muscle preparation than with soluble cytochrome *c*, but this does not exclude the possibility that in the tissues which contain this enzyme it is so situated spatially that the reaction with cytochrome *c* is accelerated. Richert, Vanderlinde & Westerfeld (1950) have recently reported experiments on the effect of Antabuse (tetraethylthiuram disulphide) on liver xanthine oxidase, which suggest that, in crude preparations, the enzyme is bound to some cell constituent and does not react readily with oxygen.

SUMMARY

1. The ability of oxidized cytochrome *c* to act as a hydrogen acceptor in the hypoxanthine-xanthine oxidase system has been confirmed.

2. In all the preparations studied the anaerobic rate of reduction of cytochrome *c* was greater than that under aerobic conditions. It is considered that oxygen and oxidized cytochrome *c* compete with one another as hydrogen acceptors in the xanthine oxidase system. This is contrary to the findings of Horecker & Heppel (1949).

3. The cytochrome *c* of heart-muscle preparation was found to be reduced by hypoxanthine in the presence of purified milk xanthine oxidase more slowly than the purified, extracted pigment.

4. Phosphate, glyoxaline and glycine buffers, pH 7·3, and sodium and potassium chloride inhibited the rate of reduction of cytochrome *c* by hypoxanthine and xanthine oxidase considerably. Phosphate buffer, pH 7·3, 3×10^{-3} M, inhibited the anaerobic reduction by 44 %.

5. The possibility that reduced xanthine oxidase is not oxidized directly by oxygen in animal tissues is discussed.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in this work and Dr E. C. Slater for helpful discussions. I also wish to thank the Australian National University for a scholarship.

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Studies in Rhodopsin

5. CHEMICAL ANALYSIS OF RETINAL MATERIAL

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The chemical composition of the specialized tissues involved in vision is not well established. In respect of low-intensity or scotopic vision, the need is for analysis of the rod outer segments and of rhodopsin, that is of the receptor end-organ and the receptor substance. Retinas themselves have been analysed for various constituents, but the picture is very incomplete and much more work is needed. Rod outer segments (cf. Lythgoe, 1937; Saito, 1938) have not until recently been separable in amounts adequate for analytical work, but appreciable quantities can now be obtained (Collins, Love & Morton, 1952).

Krause (1937) showed that rhodopsin solutions contained phosphorus and choline, suggesting the presence of lecithin. Broda, Goodeve & Lythgoe (1940) reported that, in rhodopsin solutions, 20 % of the weight of solute could be ascribed to phospholipin. Wald & Ishimoto (1946) extracted rod preparations with light petroleum until no further phosphorus-containing material went into solution. Nevertheless, on exposing the extracted rod preparation to light, phospholipin in appreciable quantity was set free, presumably as a result of the decomposition of rhodopsin, and could then be extracted.

Collins & Morton (1950*b*) suggested that nucleotides, probably as nucleic acid, were present in rhodopsin solutions. Ehrlich & Dische (1950) analysed whole retinas from several species of animals for nucleic acid and found a considerable difference from other tissues. They also found that the amounts and proportions of deoxypentose- and pentose-nucleic acids (DNA and PNA) did not vary as between dark and light adapted eyes.

It was decided, therefore, to study the distribution of phospholipin and of nucleic acid in retinas and isolated rod outer segments. For this, the fractionation procedure of Schmidt & Thannhauser (1945) has been followed.

EXPERIMENTAL

Materials. Cattle retinas were obtained from the local abattoir, and the rod outer segments were prepared as described by Collins *et al.* (1952). Some experiments were done using *Rana esculenta* imported from Holland.

Analytical methods. For the analysis of rods, a suspension was prepared and samples used for various determinations. For the estimation of the wet weight, a sample of the rod suspension was centrifuged in a weighed tube and then left to drain. In order to determine the dry weight, the tube containing the rods was dried at 100° until the weight was constant. Another sample was taken and the rhodopsin present was extracted with 1% (w/v) aqueous digitonin after treatment with 4% (w/v) K_2SO_4, $Al_2(SO_4)_3$, $24H_2O$. The amount was determined spectroscopically after mixing with an equal volume of borate buffer (0·05M, pH 9·3) (Collins & Morton, 1950*a*). The remainder of the rod suspension was used for the determination of the P fractions. For this, the fractionation procedure of Schmidt & Thannhauser (1945) was used; this yielded five fractions; acid-soluble, lipid, pentosenucleic, deoxypentosenucleic and 'phosphoprotein' P. Centrifuging was used instead of filtration. The P was estimated after $HClO_4$ digestion by the method of Berenblum & Chain (1938). The inorganic P, equivalent to the phosphoprotein in the Schmidt & Thannhauser fractionation, was determined by the method of Delory (1938).

RESULTS

Analyses of retinas and rod outer segments

The figures shown in Table 1 give the distribution of phosphorus in retinas from cattle and frogs, and also in the corresponding rod outer segments. These were prepared as described by Collins *et al.* (1952) using the sugar gradient method.

Collins & Morton (1950*b*) conclude that the 'molecular' extinction coefficient of rhodopsin is about 100000 (one 'molecule' = one chromophore) and that one chromophore contains two C_{20} units. Hence one can calculate the amount of vitamin A (mol.wt. 286) equivalent to the rhodopsin present in the solution. As will be seen from Table 1, the rhodopsin content of the rods is twice that of the whole retinas, on a dry weight basis.

The phosphorus fractions show two interesting results: (i) the amount of deoxypentosenucleic acid in rods is extremely low; this agrees with the fact that the rod outer segments have been detached from the rod nuclei; (ii) the amount of phospholipin has been concentrated in rod outer segments almost as much as the rhodopsin, suggesting that the two may be associated.

The other phosphorus fractions do not show such striking differences; the acid-soluble phosphorus and the pentosenucleic acid show decreased concentrations in rods as compared to whole retinas, whilst the 'phosphoprotein' fraction, the significance of which is not clear, is small in both cases and not much altered.

The figures for frog retinas and rod outer segments are not so complete, but are similar. By following the accepted convention that phospholipins contain 4% phosphorus, and that the deoxy-

Table 1. *Composition of retinas and rod outer segments*

(The results are expressed as mg./100 g. fresh tissue and as mg./100 g. dry tissue, shown in brackets.)

Tissue	Dry matter g./100 g. wet tissue	Rhodopsin as vitamin A*	Phosphorus fractions: Acid-soluble	Phospholipin	DNA	PNA	'Phosphoprotein'	Ratio of PNA/DNA
Cattle, whole retinas	14·1	5·5 (39)	61·2 (434)	92·5 (656)	59·7 (423)	20·9 (148)	5·5 (39)	0·35
Cattle, rod outer segments	27·2	22·6† (83)	33·5 (123)	320·0 (1108)	0·8 (3)	17·5 (64)	9·1 (33)	21·9
Ratio of the value for rods/value for whole retina	—	4·1 (2·1)	0·55 (0·28)	3·5 (1·8)	0·013 (0·007)	0·84 (0·43)	1·7 (0·85)	—
Frog, whole retinas	17·0	1·35‡ (7·9)	48·0 (282)	87·8 (517)	63·5 (374)	27·1 (160)	7·2 (42)	0·43
Frog, rod outer segments	—	—	5·54§	41·2§	5·4§	15§		2·8

* Assuming the data given by Collins & Morton (1950*b*).
† The rhodopsin solution obtained from these rods had $P_{400\ m\mu} = 31$ (Collins *et al.* 1952).
‡ Average yield from a number of separate experiments.
§ In μg. These rods were from 120 frog eyes, but owing to loss of material during preparation there was insufficient for weighing.

The amount of vitamin A was calculated as follows. The rhodopsin was extracted, as described above, from a number of whole retinas or a sample of a rod suspension, and the absorption spectrum measured before and after bleaching the solution with light. The difference between the readings at 500 mμ. is due to the rhodopsin chromophore.

pentose- and pentose-nucleic acids and the acid-soluble fractions all contain approximately 10% P, the amounts of these compounds on a dry weight basis can be calculated. The results are shown in Table 2. The most notable feature is the high proportion of phospholipin, nearly 30% in the case of the cattle rod outer segments.

Table 3 shows the distribution of phosphorus in a number of rod preparations; these have been arranged in order of the optical purity of the rhodopsin solutions prepared from the corresponding rod preparation. It is significant that the rod preparations which yield the purest rhodopsin solutions contain the lowest proportion of deoxypentose nucleic acid. As rod outer segments should contain no nuclear material, deoxypentose nucleic acid should also be absent. Its presence, therefore, indicates some contamination with nuclei. For the other constituents, the findings are inconclusive.

Table 2. *Composition of retinas and rod outer segments*

(The results are expressed as g./100 g. of dry tissue.)

	Cattle		Frog, whole retinas
	Whole retinas	Rods	
Rhodopsin (as vitamin A)	0·039	0·083	0·0079
Acid-soluble phosphorus compounds (assumed 10% P)	4·3	1·2	2·8
Phospholipin (assumed 4% P)	16·4	27·7	12·9
Deoxypentose nucleic acid (assumed 10% P)	4·2	0·03	3·7
Pentosenucleic acid (assumed 10% P)	1·5	0·6	1·6

Effect of various subsidiary treatments. Table 4 shows the effect of alum treatment on the phosphorus distribution in rhodopsin solutions. If the alum treatment is omitted, the solutions contain much less phospholipin than solutions prepared from alum-treated rods, and for pentosenucleic acid there is an even larger difference. For rods which have not been treated with alum, most of the pentosenucleic acid remains insoluble in aqueous digitonin, whereas, after treatment with alum, most of the pentosenucleic acid becomes soluble. It must be concluded that treatment with alum splits off phospholipin and pentosenucleic acid from the protein and denatures it. The resulting rhodopsin solution is, however, purer. The accompanying non-rhodopsin protein is presumably denatured and is thus less soluble in digitonin solutions.

In preliminary experiments it had been found that if cattle rod outer segments were ground with quartz, and the resulting suspension subjected to differential centrifugation, the rhodopsin was fairly evenly distributed between the fractions. It was thought worth while to examine the distribution of phosphorus in rods after differential centrifugation.

Rod outer segments obtained from sixty cattle eyes were homogenized with 5 ml. of 0·25 M-sucrose, in a glass Potter-Elvehjem homogenizer. The homogenate was then made up

Table 3. *Distribution of phosphorus in rods contaminated to varying degrees*

(*P* is the ratio of the extinction at 400 mμ. to that at 500 mμ. of the rhodopsin solution prepared from a sample of the total rod suspension. The other figures give the amount of phosphorus in the fraction as a percentage of the total phosphorus.)

P	Acid-soluble	Lipid	DNA	PNA	'Phospho-protein'
30·6 (best)	8·9	81·0	0·2	5·6	2·6
30·6	8·8	81·0	0·2	4·3	2·2
33·0	1·7	88·0	0·7	6·2	3·1
34·0	22·0	66·0	1·0	7·1	4·2
35·6	25·6	63·8	1·3	8·1	1·2
35·6	25·2	65·0	1·3	7·0	1·1
45·0 (worst)	7·9	79·6	1·6	8·7	2·1

Table 4. *Distribution of phosphorus in digitonin extracts of cattle rod outer segments*

(1% aqueous digitonin was used as extractant, 4% alum solution was used to harden some of the rods. All figures represent the P in a fraction as percentage of the total P.)

	Acid-soluble	Lipid	DNA	PNA	'Phospho-protein'	Total
Alum-treated, extract	64*		†	10·1	3·5	77·6
Alum-treated, residue	3·7	15·3	†	0·4	3·0	22·4
Not alum-treated, extract	29·8*		†	1·3	4·1	35·2
Not alum-treated, residue	5·4	45·7	†	7·1	6·4	64·6

* It proved impossible to effect a quantitative separation of the acid-soluble and lipid P in the presence of digitonin and hence the combined total is given.

† Very small—less than 0·5% of total P.

to 10 ml., and 2 ml. were removed for the estimation of the P distribution in the whole homogenate. Then 7·5 ml. were taken and centrifuged for 10 min. at 600 *g*. The precipitate was resuspended in 1 ml. of 0·25 M-sucrose and recentrifuged. The precipitate was designated fraction 1. The combined supernatants were then centrifuged for 60 min. at 2000 *g*, and the precipitate was washed with 1 ml. of 0·25 M-sucrose and recentrifuged. This precipitate was fraction 2. Fraction 3 was obtained by centrifuging for 75 min. at 10000 *g* the combined supernatant and washing from fraction 2. Fraction 4 was the supernatant from fraction 3.

The phosphorus distribution of each of these four fractions is shown in Table 5. Fraction 2 contains 61 %, and fraction 3, 28·8 %, of the total phospholipin. Fraction 4, the supernatant, contains 78·9 % of the acid-soluble phosphorus. Apart from this there appears to be no great accumulation of phosphorus compounds in any one fraction.

Two further experiments were carried out to see if it was possible to remove the phospholipin or pentosenucleic acid from rods, and if this would improve the purity of the rhodopsin solution obtained from them.

was not sedimented in 10 min. at 600 *g* but was thrown down in 60 min. at 2000 *g*, using 0·25 M-sucrose as the medium. This material was then extracted with several successive amounts of Edsall's solution for 3 hr. at room temperature and for 8·5 hr. at 0°.

The amount of pentosenucleic acid phosphorus decreased from 19·0 μg. per sample to 9·9 μg. for an equivalent sample after extraction. The rod fragments before and after treatment with Edsall's solution yielded rhodopsin to digitonin solution, but the removal of nucleoprotein did not improve the optical purity.

DISCUSSION AND CONCLUSIONS

Erhlich & Dische (1950) examined the retinas of rabbits and steers, and obtained values for deoxypentosenucleic acid of 396–1240 mg. % and for pentosenucleic acid of 95·4–232 mg. % on a wet weight basis. If it is assumed that the nucleic acids contain 10 % phosphorus, then the present results correspond to about 600 and 250 mg. % respectively

Table 5. *Distribution of phosphorus in cattle rod outer segments after homogenization*

(The rod outer segments from sixty cattle eyes were homogenized in 0·25 M-sucrose. The figures given are the amounts of P expressed as μg./retina.)

	Acid-soluble	Phospho-lipin	DNA	PNA	'Phospho-protein'	Totals
Whole homogenate	3·00	23·10	0·19	1·70	0·84	28·83
Fraction 1 (600 *g* for 10 min.)	0·07	0·86	0·04	0·20	0·08	1·25
Fraction 2 (2000 *g* for 60 min.)	0·40	13·70	0·07	0·59	0·05	14·81
Fraction 3 (10000 *g* for 75 min.)	0·26	6·50	0·04	0·38	0·07	7·25
Fraction 4 (supernatant)	2·73	1·43	0·07	0·55	0·06	4·84
Totals	3·46	22·49	0·22	1·72	0·26	28·15

The rod outer segments from twenty-nine cattle eyes were dried at $-5°$ in a high vacuum. This took about 70 min. The powder was then ground up in light petroleum (b.p. 40–60°). After an interval, the mixture was centrifuged and the solvent decanted. The dried powder was re-extracted, in this manner, seven times.

The parent rods contained 15·6 μg. phospholipin phosphorus per retina and the combined light petroleum extracts contained 2·11 μg. P per retina. The value for $P_{400\,m\mu}$ (Collins *et al.* 1952) for the rhodopsin solution, obtained by extracting the solvent-extracted rods was 79, compared with a value of 47 for the rhodopsin from untreated rods.

Nucleoprotein can be dissolved in strong salt solutions; thus Jeener (1948) extracted nucleoprotein from mitochondria using Edsall's solution (0·6 M-KCl, 0·01 M-Na_2CO_3, 0·04 M-$NaHCO_3$). It seemed worth while to try this technique on the rod outer segments especially since it was found that Edsall's solution did not destroy the rhodopsin. A preparation was obtained from cattle rod outer segments after homogenization; the fraction used for both cattle and frog retinas. This agreement is very satisfactory especially as Erhlich & Dische used a different method of estimation.

As the above authors point out, retinas seem to be very different from most other tissues. The value of the ratio PNA/DNA as given by Davidson (1950) varies from 8·1 for ox pancreas to 0·4 for thymus. Hence retinas with PNA/DNA equal to 0·4 share with thymus the lowest recorded values of this ratio. The amount of PNA phosphorus varies (Davidson, 1950) from 185 mg./100 g. to 20 mg./100 g. Again the retina with 25 mg./100 g. has nearly the lowest recorded amount of pentosenucleic acid. The figure for DNA phosphorus, 60 mg./100 g., is intermediate and represents quite an average concentration. However, these comparisons are complicated by large variations in the percentage dry matter in various tissues. The amounts of acid-soluble phosphorus and phospholipin are not greatly different from those found in liver. The amount of 'phosphoprotein' appears to be much less than in liver—about one-fourth on a dry weight basis.

The high proportion of phospholipin in rod outer segments, about 30 % on the dry weight, is noteworthy. According to Schneider (1946), rat-liver mitochondria contain 18 % on the dry weight and this appears to be the highest recorded figure. The amount of pentosenucleic acid appears to be quite low, less than 1 % on the dry weight, and it is conceivable that this represents contamination by other retinal fragments.

In order to interpret the work on isolated rod outer segments it is informative to consider the work of Sjöstrand (1949) using the electron microscope. He showed that rod outer segments of a guinea pig retina are composed of about 2000 disks, each being about 6–9 mμ. thick and 2 μ. in diameter. When rods break up, the fragments contain variable numbers of disks—from hundreds down to less than ten. Hence it would be expected that if rod outer segments were fragmented, without, however, damaging the individual disks, a continuous range of fractions would be obtained, uniform in composition. However, two other facts may modify this conclusion. The first is that there is reported to be a space between the disks which may contain water-soluble materials; secondly, nodules are apparent on the surface of the disks, which may be detached during homogenization, unless, of course, they are artifacts arising from electron microscopy.

In the present work the results of homogenization and differential centrifugation of cattle rod outer segments indicate that the four fractions obtained are not homogeneous, although the rhodopsin appears to be fairly evenly distributed. The supernatant (fraction 4) has the largest proportion of acid-soluble phosphorus, suggesting that some cytoplasmic fluid is in fact contained between the disks. Fractions 2 and 3 are similar and are notable for the fact that 90 % of the phospholipin of rods is in these two fractions. If fraction 1 contained the larger tissue fragments, such as intact rods, the rod fibres (Sjöstrand, 1949) and other contaminating material, it would seem logical to conclude that fractions 2 and 3 consisted largely of the disks, perhaps as aggregates of various sizes. In that case 90 % of the phosphorus in the disks will be as phospholipin. It was unfortunately not possible to obtain the weight of the fractions and at present it can only be said that the phospholipin, on a dry weight basis, must exceed 30 %.

The results of these chemical analyses appear to be in harmony with Sjöstrand's description of rod outer segments and suggest that the material of the disks is largely lipoprotein.

SUMMARY

1. The distribution of phosphorus compounds, acid-soluble materials, phospholipin, deoxypentose- and pentose-nucleic acids and phosphoprotein, has been recorded for cattle and frog retinas and rod outer segments.

2. Rod outer segments contain little or no deoxypentose nucleic acid and about 30 % phospholipin on the dry weight.

3. Cattle rod outer segments have been fragmented in a homogenizer and four fractions obtained by differential centrifugation. An explanation of the results is given in terms of the structure of rod outer segments proposed by Sjöstrand (1949).

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Studies in Biochemical Adaptation. The 'Mass Action' Theory of Enzyme Adaptation

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The occurrence of enzyme adaptation, known since the beginning of the century, has aroused increasing interest in the last fifteen years. Earlier 'explanations' were purely teleological until Yudkin (1938) put forward his 'mass-action' theory.

According to this theory the adaptive enzyme is formed from a postulated precursor with which it is in equilibrium. Combination of the enzyme with any substance—its substrate or a drug—upsets the equilibrium and more enzyme is produced from the precursor until balance is restored. The theory has been criticized by Spiegelman (1946) and Spiegelman & Reiner (1947) on the grounds, for instance, that it is not possible to predict either an exponential rate of adaptive enzyme formation, or competition between enzyme-forming systems. While these predictions are not directly deducible from the original theory they are certainly not in contradiction with it (see below).

It is beyond the scope of this paper to deal in detail with these criticisms or with alternative theories that have been proposed (see, for example, Monod, 1947; Northrop, 1949). However, the theories of Hinshelwood (1946) and of Spiegelman (1946) require brief mention.

Hinshelwood assumes a series of enzymes linked so that the end product of one reaction becomes the substrate for the next. Each enzyme catalyses its own formation according to the scheme

enzyme + substrate → more enzyme + products.

This implies that the enzymes of the cell, and therefore the protoplasm generally, increase exponentially. Adaptation can, however, occur in organisms which are not growing in this way—in animals (see Weinland, 1905–6; Abderhalden, 1937; Davies & Yudkin, 1951), or in washed suspensions of bacteria —and it is difficult to see how the theory would apply in these instances.

Spiegelman's theory (1946) postulates three self-duplicating units—the gene, the plasmagene and the plasmagene-substrate-enzyme complex—each producing partial or complete replicas of itself. Although the theory accounts for many of the facts it is somewhat cumbersome, and, like Hinshelwood's, does not seem applicable to enzyme adaptation in animals or in non-growing bacteria.

It would be of advantage to have a theory which would apply to animals as well as bacteria, which would explain the relevant facts simply and which would lead to qualitative and quantitative predictions by which it could be tested. It seemed that these requirements could be largely fulfilled by extending Yudkin's original 'mass action' concept to cover a wider range of biochemical reactions.

'Mass action' enzyme model

We shall examine the consequences of the general assumption that chemical equilibria exist between the various protein components of the cell, postulating also that: (*a*) proteins are synthesized from elementary substances; (*b*) the end products of an enzyme reaction may be used for the building of cell material in general and enzymes in particular.

Since no account has been taken of the important part played by genes in the control of enzymes, this model will clearly give an over-simplified picture.

The model constructed on the above premises is shown in Fig. 1. B is a pool of elementary building blocks from which proteins and enzyme precursors are synthesized. The term 'building blocks' will be held to include sources of carbon and nitrogen as well as any vitamins, inorganic ions, etc., that may be needed. The amount of material in the pool may be only a small proportion of the total cell material, but, owing to the equilibria existing, there will always be some free building material. The various cell proteins (Pr_1, Pr_2, etc.) and enzyme precursors (P_1, P_2, etc.) are synthesized from, and are in equilibrium with, the materials in B. The enzymes E_1 and E_2 are held to be formed directly from their specific precursors by a unimolecular reaction, and are shown as reacting with their substrates in the usual way. Part of the product of an enzyme reaction is contributed to B as building material while the rest is catabolized. The proportion of the product used for each of these two purposes will vary according to the nature of the product and the physiological conditions. Thus the proportion 'fed back' to B will be large or small depending on whether the organism is or is not growing.

It is important to note that the model implies a balanced metabolic system. For example, the pro-

teins Pr_1 and Pr_2 will be in a fixed ratio to one another and to all the other proteins and enzymes of the cell. The relative concentrations of the cell components will depend on the nature of the equilibria, and an alteration in the concentration of any one substance will affect the concentrations of substances in equilibrium with B. In addition, no qualitative distinction is made between 'adaptive' and 'constitutive' enzymes. Instead it is assumed that for a 'constitutive' enzyme the over-all equilibrium between B and the enzyme lies in the direction of the latter. For an 'adaptive' enzyme the opposite holds.

concentration, i.e. the enzyme is autocatalytic and its rate of increase is given by

$$\frac{de_1}{dt} = k' e_1. \qquad (1)$$

If s_1 is not large enough to be regarded as constant

$$\frac{de_1}{dt} = k e_1 s_1. \qquad (1a)$$

It may be noted in passing that these are among the fundamental equations of Hinshelwood's kinetic studies (see above).

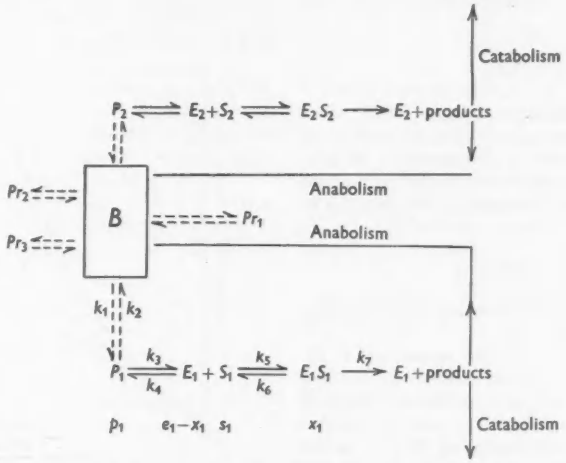

Fig. 1. Model of enzyme adaptation by 'mass action'. B, pool of building blocks; Pr_1, Pr_2, Pr_3, proteins; P_1 and P_2, enzyme precursors; E_1 and E_2, enzymes; S_1 and S_2, substrates; p_1, e_1, s_1 and x_1 are concentrations; k_1, k_2, etc. are velocity constants. Broken arrows indicate that a number of reactions may be involved.

Growth of organisms in terms of the 'mass action' model

In the growth of micro-organisms the medium becomes partly transformed into protoplasm. If S_1 (Fig. 1) is the sole source of carbon and energy, then a large part of the reaction product is ultimately contributed to the pool of 'building blocks'; either the product itself, or substances derived from it after further interaction, are 'fed back' to B. In either case, the rate at which material passes to B is proportional to the rate at which S_1 is broken down, and this in turn depends on the product of concentrations of E_1 and S_1. Now in a fresh inoculum, S_1 will be relatively so large that its concentration may be regarded as constant and the growth of B then depends directly on the concentration of E_1. From Fig. 1 it can be seen that an increase in B leads to an increase in P_1 and hence in E_1. Therefore the rate of growth of E_1 is proportional to its own instantaneous

Integration of Equation (1) indicates that the growth of E_1 will be given by an exponential equation. Furthermore, since B is available not only for the synthesis of E_1 but of protoplasm generally, it follows that the growth of the culture as a whole will be represented by a similar equation.

In animals the picture is more complicated. The growth phase may be considered as similar to that of the micro-organism in that a large proportion of the products of enzyme reactions passes to B. As the animal matures the proportion becomes smaller and smaller, and in the adult it is sufficient only to maintain the body structure.

Enzyme adaptation caused by the substrate

According to the 'mass action' theory, combination of the enzyme with its substrate disturbs the equilibrium with the precursor and more enzyme is synthesized. The process will be considered in two

situations: (*a*) Adaptation in a non-growing system, e.g. in an animal or in a washed bacterial suspension. In other words, it is assumed that there is no 'feed back' of products to B. (*b*) Adaptation accompanied by a 'feed back' of materials to B.

(*a*) *Adaptation in the absence of 'feed back'.* Let E_1 (Fig. 1) be the enzyme under consideration. The rate of formation of E_1 from P_1 is given by

$$\frac{de_1}{dt} = k_3 p_1 - k_4(e_1 - x_1). \qquad (2)$$

If enough substrate is present to saturate the enzyme

$$e_1 = x_1 \quad \text{(very nearly)},$$

and (2) becomes

$$\frac{de_1}{dt} = k_3 p_1. \qquad (2a)$$

A number of possibilities arise, some of which may now be considered.

(1) *Large concentration of the precursor.* If p_1 is large in comparison with the amount of enzyme finally formed, it may be regarded as almost constant, that is

$$\frac{de_1}{dt} = \text{constant},$$

and E_1 will appear at a linear rate until the equilibrium is restored (Fig. 2*A*).

(2) *Small concentration of the precursor.* If the initial amount of P_1 is small relative to the final amount of enzyme it will, after addition of the substrate, fall almost to zero. The rate of enzyme synthesis thereafter will depend on the rate of formation of P_1 from B. Although the amount of material in B may be small, any lowering of its concentration will be corrected by degradation of the proteins which are in equilibrium with B. If the amount of E_1 is small in relation to these proteins, B may be regarded as a reservoir whose contents are continuously adjusted to a fixed level. The rate of production of E_1 from this constant reservoir will be constant and the graph obtained will again be linear.

On the other hand, if the replenishment of B is restricted, i.e. if a building block essential for the synthesis of E_1 is limited in quantity and is becoming exhausted, there will be a continuously falling rate of enzyme formation (Fig. 2*B*). This type of curve is not likely to occur frequently for, if the building blocks needed for E_1 are constituents of normal proteins, it might be difficult to obtain enough restriction in building material to produce a noticeably falling rate of adaptation.

(*b*) *Adaptation accompanied by 'feed back'.* With bacteria, the products of the action of E_1 upon S_1 may not be entirely catabolized, especially when some alternative source of energy is present. If the 'feed back' of these products is sufficient to affect the pool of building blocks, the situation becomes similar to that discussed under the heading of bacterial growth—E_1 will increase autocatalytically and the curve obtained will be of the form shown in Fig. 2*C*. If other necessary factors are present there may be concurrent growth and cell division.

In experiments on adaptation of washed yeast cells to galactose it was found that the rate of adaptation was either linear or exponential (Type 2*A* or 2*C*). Whenever the rate was linear there was no increase either in the total count or the dry weight of the cells. An exponential rate of adaptation was usually, but not invariably, accompanied by a significant increase in one or both (Mandelstam & Yudkin, 1952*a*).

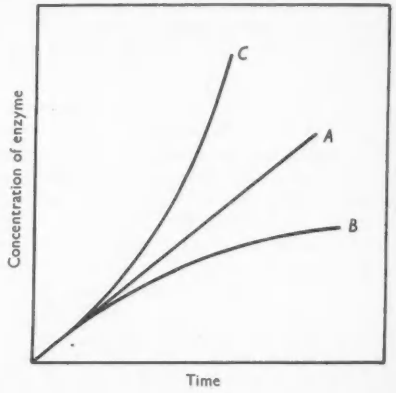

Fig. 2. Theoretical possibilities for rate of adaptation. (*A*), linear rate when final amount of enzyme formed is relatively small; (*B*), falling rate when final amount of enzyme formed is relatively large; (*C*) increasing rate when there is a continuous increase in the amount of building material.

Concentration of substrate and rate of adaptation

In the presence of substrate, enzyme formation will continue until equilibrium between free enzyme and precursor is restored; that is, it will cease when the requisite number of free enzyme molecules is present. For any particular degree of saturation, the number of molecules of free enzyme will be proportional to the total amount of enzyme present. Thus, when the degree of saturation is high and the amount of enzyme small, there will be so few free enzyme molecules that the rate of enzyme formation is nearly maximal and, at this stage, further increase in substrate concentration would not noticeably affect it. As the amount of enzyme increases, the number of free enzyme molecules increases even if the degree of saturation is unaltered. The rate of formation then begins to fall and

eventually no more enzyme is formed. Thus, if a series of concentrations of substrate is used, all of which virtually saturate the enzyme, the initial rates of enzyme formation will be identical, but the lower concentrations will allow equilibrium to be attained earlier. Thus, the final amounts of enzyme formed will depend on the substrate concentration. These predictions were confirmed in experiments on galactozymase in yeast (Mandelstam & Yudkin, 1952*a*).

Concentration of substrate and extent of adaptation

It has been indicated that the amount of enzyme finally formed depends on the amount of substrate causing the adaptation. Some expressions describing this dependence will now be derived for two experimental conditions in non-growing systems: (*a*) growth of precursor unrestricted, i.e. all the building blocks required for the production of the enzyme are amply available; (*b*) growth of precursor restricted, i.e. only a limited amount of material is available for synthesis of the precursor.

(*a*) *Growth of precursor unrestricted.* Let E be the 'adaptive' enzyme, P its precursor, B the pool of building blocks and S the substrate. We then have

$$B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E+S \underset{k_6}{\overset{k_5}{\rightleftharpoons}} ES \xrightarrow{k_7} E+\text{products}$$

$$p \qquad e-x \quad s \qquad x$$

with concentrations as shown.

The rate of change of x follows from the law of mass action and is given by

$$\frac{dx}{dt}=k_5 s(e-x)-k_6 x-k_7 x. \tag{3}$$

Initially, that is before addition of S, x will be zero. After S has been added, x will rise rapidly to a small value determined by the amount of E initially present. The further rate of increase of x will depend on the rate of synthesis of fresh enzyme. The process will continue until the system has adjusted itself by producing enough enzyme to restore the enzyme-precursor equilibrium. There will then be, for a short time at any rate, a steady state condition in which x will have reached a maximum and

$$\frac{dx}{dt}=0.$$

Whence $$k_5 s(e-x)-k_6 x-k_7 x=0,$$

or $$x=\frac{es}{s+\dfrac{k_6+k_7}{k_5}}. \tag{4}$$

The term $(k_6+k_7)/k_5$ can be replaced by a single constant which is usually denoted by K_m, the Michaelis constant. Briggs & Haldane (1925) and Haldane (1930) have pointed out that this is not strictly correct unless k_7 is negligible compared with k_6. The approximation is, however, often justified in practice and will be used here for simplicity of notation.

Equation (4) then becomes

$$x=\frac{es}{s+K_m}. \tag{5}$$

Let e_a and x_a be the steady state concentrations when $s=s_a$. Then

$$x=\frac{e_a s_a}{s_a+K_m}. \tag{6}$$

If e_b and x_b are the steady state concentrations for some other value of s $(=s_b)$

$$x_b=\frac{e_b s_b}{s_b+K_m}. \tag{7}$$

The concentration of the free enzyme will be e_a-x_a and e_b-x_b in the two cases respectively. By hypothesis, building blocks are amply available so that the concentration of the precursor is always restored finally to its initial value. If the final concentration of P is constant, the concentration of free enzyme in equilibrium with P must also be constant, and

$$e_a-x_a=e_b-x_b. \tag{8}$$

Substituting in (8) the values for x_a and x_b from (6) and (7)

$$e_a-\frac{e_a s_a}{s_a+K_m}=e_b-\frac{e_b s_b}{s_b+K_m}.$$

Whence

$$e_a\left(1-\frac{s_a}{s_a+K_m}\right)=e_b\left(1-\frac{s_b}{s_b+K_m}\right),$$

and $$e_b=e_a\left(\frac{s_b+K_m}{s_a+K_m}\right). \tag{9}$$

It follows from (9) that, if the enzyme concentration e_a is known for a particular concentration of substrate s_a and K_m is also known, then e_b can be calculated for any other value of S (i.e. s_b). The relationship between the equilibrium amounts of enzyme and substrate is a straight line cutting the vertical axis at a positive value given by

$$e_a K_m/(s_a+K_m).$$

For most 'adaptive' enzymes this value will be nearly zero (see Fig. 3*A*).

Mandelstam & Yudkin (1952*b*) have carried out experiments on the hepatic arginase of rats in which Equation (9) was applicable. Building blocks were freely available in that the animals were given a full diet, and a steady state was ensured by prolonging the experiment for 22 weeks. At this stage, growth was so slight that the effect of 'feed back' could be disregarded. Under these conditions a linear relationship was found to hold between hepatic arginase and the amount of protein in the diet.

Similar results were also obtained with galactozymase in *Saccharomyces cerevisiae* (Mandelstam & Yudkin, 1952*a*).

(*b*) *Growth of precursor restricted.* By hypothesis only a limited amount of precursor can be synthesized. As a simplifying assumption it will be taken that the total amount of P that can be synthesized is present from the start. We then have

$$P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E+S \underset{k_6}{\overset{k_5}{\rightleftharpoons}} ES \xrightarrow{k_7} E+\text{products}.$$

$$p \quad e-x \quad s \quad x$$

The rate of change of p is given by

$$\frac{dp}{dt}=k_4(e-x)-k_3p.$$

When the system has reached the steady state condition

$$\frac{dp}{dt}=0,$$

and

$$x=e-p\frac{k_3}{k_4},$$

or

$$x=e-K_ep, \tag{10}$$

where K_e is the equilibrium constant for the reaction between enzyme and precursor. Since p cannot be increased it follows that

$$e+p=\text{constant}.$$

But from (5)

$$x=\frac{es}{s+K_m}.$$

Substituting this value of x in (10) we find that in any steady state

$$K_ep=e-x=e\left(1-\frac{s}{s+K_m}\right). \tag{11}$$

Whence

$$K_e(e+p)=e\left(1+K_e-\frac{s}{s+K_m}\right). \tag{12}$$

But $e+p$ is constant, so if s_a and s_b are the substrate concentrations in two steady states and e_a and e_b the corresponding enzyme concentrations,

$$e_a\left(K-\frac{s_a}{s_a+K_m}\right)=e_b\left(K-\frac{s_b}{s_b+K_m}\right), \tag{13}$$

where

$$K=K_e+1.$$

Rearranging (13)

$$e_b=e_a\left(\frac{K-\dfrac{s_a}{s_a+K_m}}{K-\dfrac{s_b}{s_b+K_m}}\right). \tag{14}$$

This equation provides a means of obtaining the equilibrium constant for the precursor-enzyme reaction. Thus, if two concentrations of enzyme (e_a and e_b) are determined for two concentrations of substrate and if K_m is known, K can be calculated. Now it will be remembered that

$$K_e=K-1,$$

where K_e is the equilibrium constant for the reaction between enzyme and precursor. For the purposes of calculation it was taken that all the precursor that could be synthesized was present from the start.

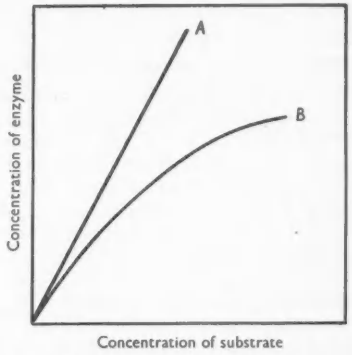

Fig. 3. Theoretical relationship between concentration of substrate and final amount of enzyme formed. (*A*), building material unrestricted; (*B*), building material restricted.

It would probably be more correct to assume that P is not present in full amount initially, but that it is continuously formed from B during the adaptation period. In that case K_e would characterize the overall equilibrium between E and the pool of building blocks, i.e. it would apply to the reaction

$$B \rightleftharpoons E.$$

On this basis, K_e becomes applicable to the problem of competition between enzyme-forming systems (see below).

Table 1. *Relationship between galactose concentration and the amount of galactozymase formed when building blocks are restricted*

(The calculated values were obtained from Equation 14.)

Galactose concentration (%)	Activity of galactozymase	
	Experimental	Calculated
2	16	17
3	26	24
4	30	30
5	36	35
6	40	39
8	47	47

Returning to Equation (14), it can be seen that if e_a has been determined for one concentration of substrate s_a, then e_b can be calculated for any other value of S (s_b). The relationship between substrate

concentration and extent of adaptation given by this equation is a curve of the type shown in Fig. 3*B*.

The galactozymase formed in washed suspensions of *S. cerevisiae* was found to be related to the galactose concentration in this manner (Mandelstam & Yudkin, 1952*a*).

In Table 1 the theoretical amounts of enzyme calculated from (14) are compared with the experimental values.

Competition between enzyme-forming systems

Qualitative considerations. The formation of an adaptive enzyme may be retarded or reversed by the presence of the 'normal' substrate (Dienert, 1901; Stephenson & Yudkin, 1936). It has also been found that, in certain conditions, one enzyme may be synthesized at the expense of another (Spiegelman & Dunn, 1947). Monod (1947) has shown that if an organism, capable of utilizing two sugars, S_1 and S_2, is inoculated into a medium containing both, the type of growth curve observed depends on the nature of the relevant enzymes E_1 and E_2. If E_1 and E_2 are both 'adaptive' or both 'constitutive' the two sugars are metabolized simultaneously and there is a single growth phase. But if E_1 is 'constitutive' and E_2 'adaptive' there are two successive growth phases. In the first phase S_1 is selectively attacked until it is completely expended. There is then a lag period at the end of which S_2 is used. This effect, which Monod has called diauxie, is found even when the cells have been previously adapted to S_2.

Let us now examine the question of competition between enzyme-forming systems on the basis of the 'mass action' enzyme model. It is necessary to stress again that 'constitutive' and 'adaptive' enzymes are assumed to differ quantitatively only. By reference to Fig. 1 a number of deductions will be seen to follow directly.

(1) The enzymes E_1 and E_2 are in equilibrium with one another through B. If a building block, needed by both enzymes, is restricted and the organism is then forced to produce E_1 adaptively, it can only do so at the expense of E_2 and of the other proteins in equilibrium with B. The extent to which E_2 will be drawn upon, rather than Pr_1, Pr_2, etc., will depend on the equilibria involved. It also follows that this competitive effect would be reduced by providing more of the building block in question.

(2) If both S_1 and S_2 are present the relative amounts of E_1 and E_2 will depend, other things being equal, on the overall equilibria between these enzymes and the pool of building blocks. If E_1 and E_2 are both 'constitutive' or both 'adaptive' their equilibrium constants, and hence their concentrations, will be of the same order, and both substrates will be metabolized simultaneously. On the other hand, if E_1 is 'constitutive' and E_2 is 'adaptive', the former will be at an advantage in the competition for building blocks, and the concentration of E_2 will remain low. Even if the cells have been previously adapted to E_2, the addition of S_1 will establish a drain on B and hence on E_2, which will then become depleted.

(3) It has so far been assumed that S_1 and S_2 are related substances (e.g. sugars). From the model it is clear, however, that any two enzymes may become involved in competition for building material. On this basis it becomes possible to explain the fact that a carbohydrate may inhibit the formation of a proteolytic enzyme (for examples of this see Gale, 1943).

Competition between enzyme-forming systems. Quantitative formulation for non-growing organisms

For purposes of calculation the simplest case only will be taken. It will be assumed that (*a*), only one building block, Q, is the subject of competition between E_1 and E_2; (*b*), the full amount of Q that can be drawn upon is present from the start; (*c*), the reactions involved are unimolecular (or pseudo-unimolecular).

No restricting assumption is necessary as to the 'constitutive' or 'adaptive' nature of either E_1 or E_2, nor are the substrates necessarily related.

In the absence of both substrates the situation may be represented as follows:

$$E_2 \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} Q \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_1.$$

After addition of the substrates S_1 and S_2 we have

$$E_1 + S_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_1S_1 \xrightarrow{k_5} E_1 + \text{products}$$

$e_1 - x_1 \quad s_1 \qquad\qquad x_1$

$k_1 \Big\| k_2$

$q \quad Q$

$k_1' \Big\| k_2'$

$$E_2 + S_2 \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} E_2S_2 \xrightarrow{k_5'} E_2 + \text{products}$$

$e_2 - x_2 \quad s_2 \qquad\qquad x_2$

Under certain conditions (see below) a steady state will be reached in which both enzymes will be in equilibrium with Q. At this stage

$$k_1 q = k_2(e_1 - x_1).$$

But $$x_1 = \frac{e_1 s_1}{s_1 + K_{m_1}} \quad \text{and} \quad \frac{k_1}{k_2} = K_e,$$

and so $$q = \frac{e_1 K_{m_1}}{K_{e_1}(s_1 + K_{m_1})}. \qquad (15)$$

A similar equation holds for the second enzyme. Hence

$$\frac{e_1 K_{m_1}}{K_{e_1}(s_1+K_{m_1})}=\frac{e_2 K_{m_2}}{K_{e_2}(s_2+K_{m_2})},$$

or

$$\frac{e_1}{e_2}=\frac{K_{e_1}K_{m_2}(s_1+K_{m_1})}{K_{e_2}K_{m_1}(s_2+K_{m_2})}. \qquad (16)$$

We thus have an expression for the relative concentrations of the two enzymes in terms of their Michaelis and equilibrium constants and the concentrations of the substrates. The equation indicates that if s_2 is kept constant, the ratio e_1/e_2 will vary linearly with s_1.

Equation (16) can, however, be quantitatively applied only in restricted conditions, for it was derived on the assumption that both enzymes reach a steady state condition at the same time. In general, this is likely to occur much more slowly than when only one enzyme is involved. Thus, it is probable that one enzyme, E_1 say, will increase more rapidly and will reach a maximum first. The second enzyme, E_2, will then have to acquire material from a depleted pool of building blocks. Its formation will accordingly be retarded and may be very slow if much E_1 has already been formed. Nevertheless, E_2 will increase, removing further material from the pool, thereby upsetting the equilibrium with E_1 which will then be diminished. Consequently, the ratio e_1/e_2 will vary continuously, but will tend to approach a limiting value defined by Equation (16). The greater the concentration of the substrates, and the greater, therefore, the adaptive response, the greater will be the depletion of building material and the more slowly will the limiting value be approached.

Bearing these considerations in mind, the following deductions can still be made. In an equimolecular mixture of S_1 and S_2, and assuming K_{m_1} and K_{m_2} to be of the same order, it follows that the ratio e_1/e_2 will depend on the ratio K_{e_1}/K_{e_2}. When E_1 is 'adaptive' and E_2 is 'constitutive' e_1/e_2 will be very small because K_{e_1}/K_{e_2} is very small (certainly not greater than 10^{-2}, and in most instances probably far less). It is clear that s_1/s_2 has to be made very large before any measurable adaptation can occur. If S_2 is present in any reasonable quantity, S_1 would have to be raised to a concentration beyond the physiological limits of the organism. In other words S_2, the 'normal' substrate, acts as an efficient inhibitor of adaptation.

Under these conditions, i.e. when one enzyme is 'adaptive' and the other 'constitutive', Equation (16) has no practical value. If, however, E_1 and E_2 are both 'adaptive' (K_{e_1} and K_{e_2} are of the same order) it will be possible to obtain measurable variations in e_1/e_2 by altering the relative concentrations of the two substrates. An increase in s_1/s_2 should cause an increase in e_1/e_2.

These predictions were shown to hold for the maltozymase and galactozymase systems of *S. cerevisiae* (Mandelstam & Yudkin, 1952*a*). Thus, it was found that the observed variations in e_1/e_2 corresponded with those calculated from Equation (16) provided that the concentrations of the sugars were low. Furthermore, an increase in the ratio of galactose to maltose produced a linear increase in the ratio of the enzymes.

SUMMARY

1. A model of enzyme adaptation by 'mass action' is proposed mainly on the assumption that enzymes and other proteins are in equilibrium with one another through a common pool of building material.

2. Qualitative and quantitative predictions have been made for the rate of adaptation, the relationship between the concentration of the substrate and the extent of adaptation, and competition between enzyme-forming systems.

3. The conformity of the predictions with experimental results has been shown in experiments described in the two following papers.

4. The extended 'mass action' theory developed in this paper is at this stage presented as no more than a working hypothesis, developed from a limited set of assumptions, to account for the main facts of enzyme adaptation in both animals and micro-organisms. It is not claimed that the conformity of experimental results with the theoretical predictions is a 'proof' of the theory, to the exclusion of other possible explanations.

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Studies in Biochemical Adaptation. The Effect of Variation in Dietary Protein upon the Hepatic Arginase of the Rat

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Much of the recent work on enzyme adaptation has been carried out in micro-organisms. Relatively little has been reported on enzyme adaptation in mammals and further work on this subject is desirable. In particular, a quantitative study would be of interest as a test of the predictions made from the extended mass action theory, which should apply to enzyme adaptation in animals as well as in micro-organisms (Mandelstam, 1952).

The enzyme chosen for study was hepatic arginase in the rat and the effect was investigated of variation in dietary protein.

Previous work in this field is inconclusive. Baldwin (1935) reported that there seemed to be a decrease in arginase in the hepatopancreas of the snail during starvation; Baldwin & Yudkin (1939), however, found such a great variation in the activity of the enzyme in different specimens that it was not possible to draw any definite conclusion concerning the effect of starvation. Seifter, Harkness, Rubin & Muntwyler (1948) reported a decrease in hepatic arginase in rats fed on a protein-free diet for 1–3 weeks. Lightbody & Kleinman (1939) found that in rats fed on diets containing 6, 25, 60 and 75% protein, the amount of enzyme in unit weight of liver was higher with higher amounts of dietary protein. It is possible that the rise in enzyme was due, at least in part, to a general increase in hepatic protein which was not estimated. Folley & Greenbaum (1946) found, with rats, that a diet containing 50% protein resulted in a higher concentration of hepatic arginase than one containing 20% protein though, with the small number of animals studied, the difference was not statistically significant. Kochakian, Bartlett & Moe (1948) estimated hepatic arginase in rats fed on a diet containing either no protein or 80% protein. After 7 days, there was a fall in the arginase in the former, partly due to a general decrease in hepatic protein, and a slight rise in the animals fed on the high protein diet, which the authors do not consider noteworthy.

Hepatic arginase is of interest in relation to the theory of Krebs & Henseleit (1932) that urea is formed in the liver through a series of reactions involving this enzyme. Although this theory has been the subject of some criticism (e.g. Bach, 1939; Trowell, 1942), it is now accepted by the majority of workers. An increase in dietary protein, and so an increase in production of urea, might well, therefore, cause an increase in the arginase involved in the process. The general problem of the use of enzyme adaptation in studies of metabolic pathways is dealt with elsewhere (see, for example, Yudkin, 1952; Davies & Yudkin, 1951).

EXPERIMENTAL

Animals. The rats were bred in this laboratory and were of an albino strain which we have elsewhere designated as *KC* 1 (Wiesner & Yudkin, 1951). Preliminary tests showed that male animals have some 15% more hepatic arginase than female animals. In the detailed experiments to be reported, only male animals were used.

Diet. From weaning to the beginning of the experiment, the animals were fed a mixed diet of cubes with additional milk and green vegetables (see Wiesner & Yudkin, 1951). This diet contains approx. 20% protein. The experimental diets containing varying amounts of protein were made according to Table 1. Animals were distributed so that one from each litter was given each of these purified diets. Food and water were given *ad lib.* The animals were weighed twice weekly.

Estimation of arginase. The animals were killed by a blow on the head and the whole liver removed and weighed. Two samples, each of about 20 mg., were accurately weighed on a glass cover-slip. Nitrogen was estimated in these samples by the Kjeldahl method. For the estimation of arginase, about 0·5 g. of liver was accurately weighed and homogenized in a Waring Blendor with 300 ml. distilled water for 90 sec. Into a Warburg cup were placed 1 ml. liver homogenate,

1 ml. water and 0·5 ml. 0·5M-phenolsulphonate-phosphate buffer, pH 8·4 (Hunter & Downs, 1944). A solution of 10 mg. arginine in 0·5 ml. water was placed in the side arm of the cup and the contents mixed after 10 min. at 38°. After a further 30 min., the contents of the cup were washed into 1 ml. 40% trichloroacetic acid, made up to 15 ml. with water and filtered. A portion of 5 ml. was taken for estimation of urea by the urease-aeration method. Urea production in these conditions was directly proportional to the amount of arginase present up to values appreciably higher than those encountered in our experiments.

Urease with no argininolytic activity was prepared from soya flour as follows: 50 g. soya flour was shaken with 200 ml. distilled water for 10 min., centrifuged and the supernatant fluid, containing largely inactive material, discarded. The flour was then extracted three times for an hour each with distilled water, the supernatant fluid from centrifugation being preserved each time. A final centrifugation was made to remove further inactive suspended material and the supernatant fluid then added to twice its volume of 96% ethanol. The precipitate was washed twice with ethanol and finally with ether. The paste was dried on trays at room temperature and finally in a vacuum desiccator. It was kept in a refrigerator and retained its activity for at least a year. For use, 1 g. of the dried preparation was finely ground and 40 ml. water slowly added with further grinding. To this was added 60 ml. glycerol and the mixture stored on ice. This preparation was stable for several weeks and 1 ml. was capable of converting about 3·5 mg. urea in 1 hr. Since the urea produced by arginase in our experiments was never more than one-tenth of this, this preparation of urease was considered satisfactory.

Enzyme activity is expressed as mg. urea N produced in 30 min. at 38° and pH 8·4.

RESULTS

Effect of amount of dietary protein on hepatic arginase

Four rats from each of eight litters were taken at the age of 5 weeks, when they weighed 60–70 g. One animal from each litter was placed on each of four experimental diets, containing 8, 17, 33 and 67% protein (Table 1). The animals on the diet with 8% protein did not thrive and although the protein was increased to 10% after 4 weeks, six of them died in the next few weeks. All the remaining animals were sacrificed after 22–24 weeks of experimental feeding.

Growth. The growth of the animals on the higher protein levels is given in Fig. 1. The most rapid growth was shown by animals on 33% protein and the slowest on 67% protein. The group consuming 17% protein grew slower initially, but by 19 weeks their weight equalled that of the intermediate group.

Table 1. *Composition of purified diets*

(To 100 g. diet was added 10 ml. vitamin solution, 1 l. of which contained: aneurin 50 mg.; riboflavin 300 mg.; choline chloride 10 g.; inositol 2·2 g.; nicotinic acid 1·0 g.; calcium (+)-pantothenate 1·0 g.; pyridoxin 30 mg.; biotin 2 mg.; 5% acetic acid 300 ml.; ethanol 100 ml. Twice weekly, each animal received three drops of cod liver oil.)

Diet	1	2	3	4
	Wt. (g.)			
Casein (Glaxo light white)	10	20	40	80
Sucrose	90	80	60	20
Arachis oil	15	15	15	15
Salt mixture	5	5	5	5

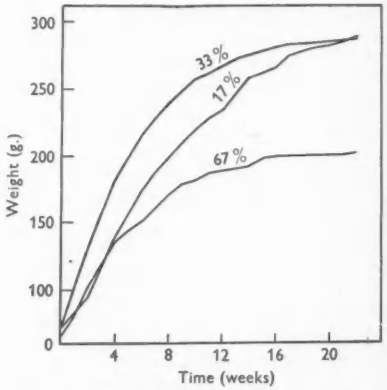

Fig. 1. Growth of rats on purified diets containing varying amounts of protein.

Enzyme activity. Table 2 gives the data which include the total amount of enzyme and the relative amount in relation to the weight of the liver, hepatic nitrogen and weight of the animal. The results for the animals on the lowest amount of protein are not included since only two animals survived, though it might be mentioned that the amount of enzyme was

Table 2. *Effect of variation in dietary protein upon hepatic arginase*

	Diet 2 (17% protein)	Diet 3 (33% protein)	Diet 4 (67% protein)
Body weight (g.)	299±7·4	279±7·1	213±3·8
Weight of liver (g.)	10·3±0·24	10·3±0·36	7·6±0·17
Hepatic N (% of wet wt.)	2·9±0·068	3·0±0·080	3·3±0·098
Hepatic arginase/mg. tissue	34·7±1·03	41·6±1·24	59·2±0·86
Hepatic arginase/mg. N	12·0±0·43	13·9±0·34	19·0±1·36
Total arginase × 10^{-4}	35·8±2·22	43·2±2·54	45·1±1·16
Total arginase × 10^{-4}/g. body wt.	0·119±0·0048	0·150±0·0059	0·212±0·0014

lower than in any other groups (e.g. 29 units/mg. liver compared with 34·7 units in animals on 17% protein).

Figures constructed from these results show the increase in enzyme with increase in dietary protein (Fig. 2). This increase is linear for enzyme concentration related to weight of liver and to hepatic nitrogen. There was also a linear increase in enzyme related to body weight, but since the absolute weight of the body, and of the liver, fell with high dietary protein, it follows that the total amount of enzyme in each animal did not rise linearly. From the functional point of view, it is of more interest to relate the amount of enzyme to unit weight of animal or of liver tissue, since the liver is the main site of urea formation.

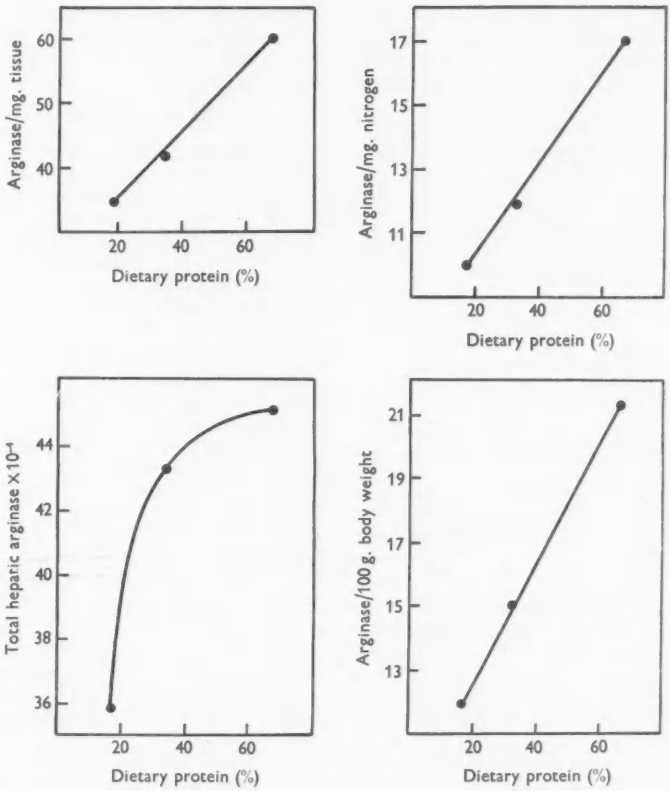

Fig. 2. Effect of variation in dietary protein on hepatic arginase. Individual graphs show enzyme activity related to weight of tissue, nitrogen of tissue, total liver and weight of animal. Arginase activity expressed as mg. urea N liberated in 30 min. at 38° and pH 8·4.

Rate of adaptation and reversibility of adaptation in young and adult rats

Groups of twenty-four young and thirty-eight adult male rats were available. They had been weaned at 3 weeks on the stock diet described above. Sixteen of the younger animals and fifteen of the older animals were placed on a purified diet containing 67% protein when they were 8 weeks and 26–28 weeks old respectively, and weighed an average of 130 and 270 g.; the remaining animals were kept on the stock diet and served as controls. At intervals over 2 weeks for the younger animals, and 3 weeks for the older animals, hepatic arginase was estimated in both the control and the experimental animals. The results are reported as if they

were obtained at weekly intervals, although the actual analyses were carried out at 5–9 and 12–16 days and on the nineteenth day.

The remaining animals on the high protein diet were then placed back on the stock diet—four young rats after 2 weeks and eight adult rats after 3 weeks. After a further 4 and 3 weeks respectively, these animals were also used for determination of hepatic arginase.

Growth. In both groups of animals, there was a loss of weight after transfer to the high protein diet (Fig. 3). Recovery began at the end of 1 week in the younger rats and at the end of a fortnight in the older rats.

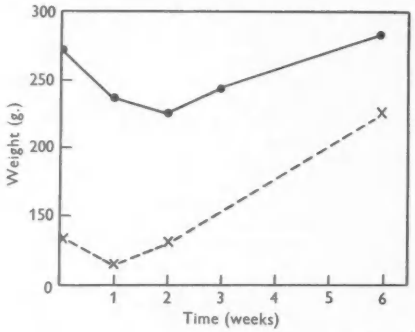

Fig. 3. Growth of rats on purified diet and on subsequent return to stock diet. ●—●, adult animals on purified diet for first 3 weeks; ×---×, young animals on purified diet for first 2 weeks.

Enzyme activity. Results were calculated in the same way as in the previous experiment. For simplicity, they are shown only in the form of graphs (Fig. 4).

The concentration of enzyme in unit weight of liver was initially lower in the young rats than in the older rats but rose higher during adaptation. Reversion to the stock diet resulted in a fall in both, but the final value, as expected, did not fall to the original low value in the younger rats, which by now had grown considerably and weighed over 200 g.

The curve relating arginase to hepatic nitrogen shows that the initial gain in enzyme in adult rats was only due to a relative increase in hepatic nitrogen. In the younger rats, however, there was a real gain in enzyme in this period, and the total gain in the second week was greater than that of the adult rats. A similar result is seen in the amount of enzyme related to body weight, and here it is observed that reversion on the stock diet is complete in both groups. The total amount of enzyme shows a regular rise and fall with young animals put on the high protein diet and then back on the stock diet. With the older animals, there was a fall in the first week which was no doubt due to the fact that the animals were in a state of semi-starvation as shown by the considerable loss in weight.

During the same period of 6 weeks, the adult control animals showed no change, whilst the young control animals showed only the increase in enzyme expected during that period of growth. For example, the arginase per mg. liver for the adult rats was 34 and 33 units at the beginning and end of the experiments, whilst for young rats it was 28 and 34 units.

DISCUSSION

The results show that an increase in dietary protein is followed by an increase in arginase, which seems to be more or less complete within 3 weeks; reversion to the previous diet results in a decrease of the enzyme to its normal values. A possible explanation of adaptation to an increase in dietary protein is that the larger amounts of protein being metabolized involve a greater use of the enzyme in producing urea. According to the mass action theory of enzyme adaptation, more arginase would be produced if more of the enzyme is in a combined form. This might occur with a high protein intake because of a greater formation of ammonia, so that more is passing through the arginine cycle, more arginine is present and hence more of the enzyme is combined. It is also possible that the substances combining with the enzyme are derived directly from the protein; these would include arginine itself and also ornithine and lysine which are known to act as competitive inhibitors for arginase (Hunter & Downs, 1945). Other explanations may be advanced for the increase of arginase with increase in dietary protein, for example, a general increase in hepatic enzymes. Although this seems unlikely, since there was no appreciable increase in hepatic nitrogen, such a possibility cannot at present be excluded.

The results are also interesting from the quantitative point of view. We have seen that the concentration of enzyme produced is directly proportional to the amount of dietary protein and that this proportionality is linear. The mass action theory of enzyme production originally suggested in respect to adaptation in micro-organisms (Yudkin, 1938) should apply equally to enzyme adaptation in multicellular organisms (Mandelstam, 1952). The linear relationship between dietary protein and hepatic arginase is in conformity with the quantitative deductions from that theory for enzyme adaptation in higher organisms and, in certain conditions, in micro-organisms. This matter is dealt with in the accompanying papers (Mandelstam, 1952; Mandelstam & Yudkin, 1952).

SUMMARY

1. A study has been made in rats of the effects of variation in dietary protein upon hepatic arginase.

2. The amount of the enzyme, both absolute and in relation to unit weight of liver, hepatic nitrogen and body weight, increases with increase in dietary protein and reverts to normal with reversion to a normal diet.

3. The adaptation to the increase or decrease in dietary protein is almost complete within 2 or 3 weeks of changing the diet.

4. The rate and extent of increase of enzyme is greater in young animals than in older animals.

5. There is a linear relationship between dietary protein and amount of enzyme produced. This is in conformity with the mass action theory of enzyme adaptation.

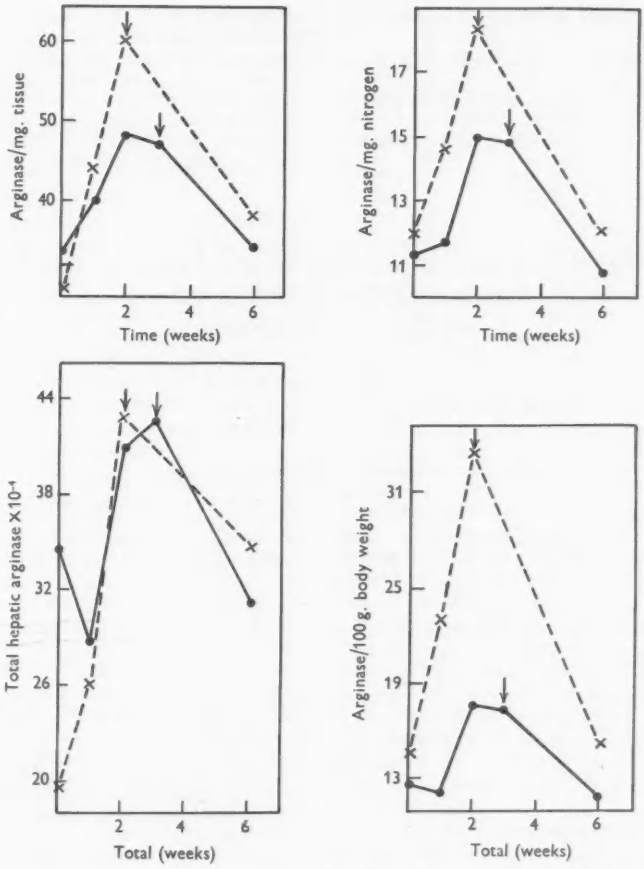

Fig. 4. Reversible adaptation of hepatic arginase. Arrows indicate return from purified diet (67% protein) to stock diet (20% protein). ●—●, adult animals; ×---×, young animals. Individual graphs show enzyme activity related to weight of tissue, nitrogen of tissue, total liver and weight of animal. Arginase activity expressed as mg. urea N liberated in 30 min. at 38° and pH 8·4.

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Studies in Biochemical Adaptation. Some Aspects of Galactozymase Production by Yeast in Relation to the 'Mass Action' Theory of Enzyme Adaptation

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The experiments to be described were designed to test some of the predictions of the extended 'mass action' theory (Mandelstam, 1952). The enzyme system chosen for study was that involved in the fermentation of galactose by yeast cells. Several enzymes are involved in this reaction, at least two of which are adaptive, so that it is not perhaps the ideal enzyme system for such a study (Stanier, 1951). It has, however, been the subject of so much work by such investigators as Stephenson & Yudkin (1936) and Spiegelman (1946), that it was nevertheless chosen for this work. For simplicity, we shall speak as if one enzyme, galactozymase, is involved.

The predictions selected to be tested were:

(1) The rate of adaptation in the presence of a given amount of galactose.

(2) The effect of substrate concentration on the rate of adaptation.

(3) The effect of substrate concentration on the amount of enzyme formed.

(4) The relative amounts of two adaptive enzymes (galactozymase and maltozymase) formed in the presence of varying amounts of both substrates.

The detailed theoretical treatment for each of these situations is given by Mandelstam (1952). A simpler diagram is given here (Fig. 1) to facilitate the necessary reference to the theory.

A general description will first be given of the techniques used. A summary of the expectations from the theory for each of the selected situations, the experiments designed to test them, and the results obtained will then be presented in order.

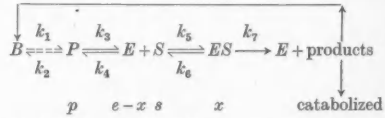
k_1 k_3 k_5 k_7
$B \rightleftharpoons P \rightleftharpoons E + S \rightleftharpoons ES \longrightarrow E + \text{products}$
k_2 k_4 k_6
p $e-x$ s x catabolized

Fig. 1. Simplified diagram illustrating mass action theory of enzyme adaptation. *B*, pool of building blocks; *P*, enzyme precursor; *E*, enzyme; *S*, substrate. Concentrations are denoted by small letters. k_1, k_2, etc., are velocity constants. Broken arrows indicate that a number of reactions may be involved.

METHODS

The organism used was a pure strain of *Saccharomyces cerevisiae* (N.C.T.C. No. 815). It was maintained on slopes, the medium containing bacto-peptone 2%, yeastrel 3%, glucose 4%, agar 1·5%. The pH was adjusted to 6·0 and the organisms grown at 29°. Cultures for the experiments were made in a synthetic medium containing 50 g. glucose, 4·7 g. $(NH_4)_2SO_4$, 750 mg. KH_2PO_4, 100 mg. $MgSO_4$, 6·6 mg. inositol, 3 mg. nicotinic acid, 3 mg. calcium (+)-pantothenate, 150 μg. aneurin, 900 μg. riboflavin, 240 μg. pyridoxin, 6 μg. biotin and water to 1 l. The pH was adjusted to 6·0 and the organisms were grown at 29° for 40–48 hr. The cultures were centrifuged and the cells washed twice in saline and suspended in galactose containing phosphate buffer ($1{\cdot}67 \times 10^{-2}$ M, pH 6·0). The suspensions were made up so that 1 ml. contained about 30 mg. dry weight of cells.

Enzyme activity was measured anaerobically in the Warburg apparatus at 38° with 3 ml. samples and expressed as μl. CO_2 evolved in 10 min. Cell counts were made in a standard blood count chamber. Dry weights of cells were determined by washing 3 ml. samples of suspension three times with distilled water by centrifugation, and drying to constant weight at 110°.

Since all samples of galactose purchased were found to contain small quantities of material fermentable by unadapted yeast cells, the galactose was purified by the method of Stephenson & Yudkin (1936).

EXPERIMENTAL

Rate of adaptation in the presence of a given concentration of substrate

From the theory, two situations are likely to occur in practice, though a third may arise in certain circumstances.

(*a*) Linear rate of enzyme adaptation. This should occur if the amount of precursor, or of building blocks, is comparatively large, and the products of enzyme action are not used for making more precursor.

(*b*) Exponentially increasing rate of enzyme adaptation. This should occur if the products of enzyme action are 'fed back' to the pool of building blocks and are used for making more precursor.

Experiments. A culture of yeast was grown in 2 l. of inorganic medium containing 5% glucose for 40–48 hr. At the end of this time, the cells were centrifuged and washed twice by suspending in saline and centrifuging. They were then suspended in 50 ml. of 5% galactose in phosphate buffer ($1{\cdot}67 \times 10^{-2}$M, pH 6·0) and incubated at 29°. After about 15 hr., 3 ml. portions were placed at intervals in Warburg flasks and the evolution of CO_2 measured. Counts and determinations of dry weight were made at the beginning and end of the period of adaptation.

Results. Altogether fifteen experiments of this nature were carried out.

In six experiments, the rate of production of enzyme was linear (Fig. 2). In each case, there was no detectable increase in the number of cells or in their dry weight. In nine experiments, the rate of production of enzyme increased exponentially (Fig. 3). Of these, seven showed a significant increase in the number of cells. The increase in weight of cells was always proportionately less, as the cells were much smaller. In three of the seven instances there was a significant increase in the dry weight of the cells. The fact that there was no such increase in the remaining experiments showing an exponential rate of adaptation can be explained along the lines suggested by Mandelstam (1952).

Conclusions. The theory predicts that the rate at which adaptation occurs is likely to be either linear or exponential. Both of these forms of adaptation curves have been obtained, although we have not yet found the experimental factors which determine which form the curve will take. In particular, a linear rate of enzyme adaptation should not occur with increase in cell weight and we have found this to be the case.

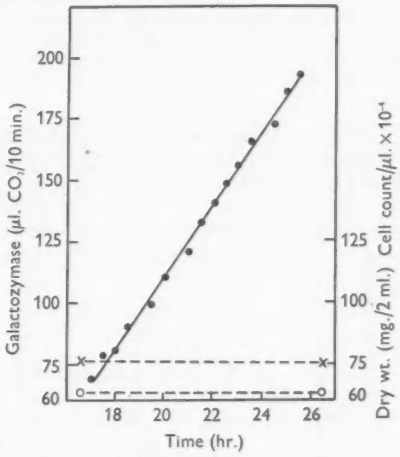

Fig. 2. Rate of production of galactozymase. Yeast cells grown in glucose medium and suspended in buffer containing 5% galactose. Rate of enzyme production constant, ●—●; cell count constant, ×---×; weight of cells constant, ○---○.

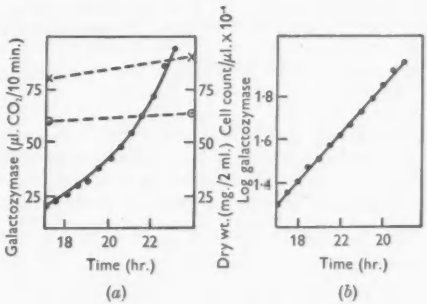

Fig. 3. Rate of production of galactozymase. Yeast cells grown in glucose medium and suspended in buffer containing 5% galactose. (*a*) Rate of enzyme production continuously increasing, ●—●; cell count increasing, ×---×; weight of cells increasing, ○---○. (*b*) Same experimental data plotted to show that increase of enzyme is logarithmic.

Effect of substrate concentration on rate of adaptation

The theory requires that the initial rate of enzyme adaptation should be independent of substrate concentration over a wide range.

Experiments. A culture was grown for 40 hr. in the inorganic medium with 5% glucose, washed twice and suspended in saline. Samples (5 ml.) of suspension were added

to flasks containing 10 ml. of phosphate buffer with galactose added to give concentrations of 3–8 % of the sugar. This range was chosen to ensure, at the lower level, that the maximal rate of enzyme production would be maintained for a sufficient time (see Mandelstam, 1952) and, at the higher level, that the cells were not damaged. The flasks were left at 29° and some of the suspension was removed at intervals for determination of galactozymase in the Warburg apparatus.

Results. The amount of enzyme formed after 22 hr. was approximately the same at all concentrations of galactose above 3 % (Fig. 4).

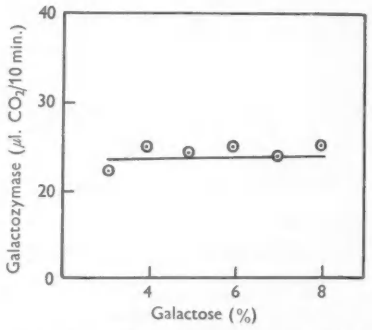

Fig. 4. Rate of production of galactozymase with varying concentrations of galactose. Yeast cells grown in glucose medium and suspended in buffer containing varying concentrations of galactose. Constant amount of enzyme formed after 22 hr. adaptation with galactose from 3 to 8 %.

Conclusions. As predicted, the rate of adaptation is initially independent of the concentration of the substrate. Later the total amount of enzyme formed increases with increasing concentration of the substrate (see below).

The effect of substrate concentration on amount of enzyme formed

The theory forecasts that the final amount of enzyme formed will depend on the substrate concentration. The quantitative relationship between the amount of enzyme and substrate concentration depends on whether or not there is an ample supply of building blocks.

(*a*) Building blocks unrestricted. If e_a is the equilibrium concentration of enzyme produced in response to substrate concentration s_a, then the concentration of enzyme produced in response to another concentration of substrate s_b is given by

$$e_b = e_a\left(\frac{s_b + K_m}{s_a + K_m}\right), \qquad (1)$$

where K_m is the Michaelis constant.

This implies a linear relationship between the substrate concentration and the amount of enzyme formed.

(*b*) Building blocks restricted. The corresponding equation is

$$e_b = e_a\left(\frac{K - \dfrac{s_a}{s_a + K_m}}{K - \dfrac{s_b}{s_b + K_m}}\right), \qquad (2)$$

where K is a constant.

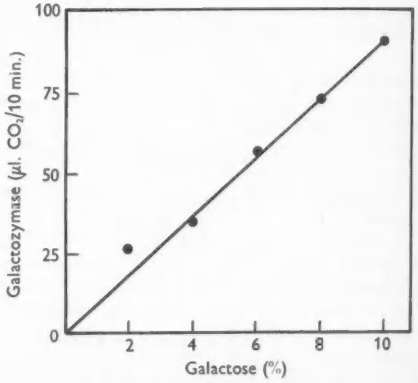

Fig. 5. Effect of galactose concentration on amount of galactozymase formed. No restriction of 'building blocks'. Yeast cells grown in media containing varying amounts of galactose. Galactozymase estimated in washed cells after 36 hr. of growth. Points give experimental results; line calculated from theory (Equation 1), using experimental value found with 10 % galactose.

(*a*) *Building blocks unrestricted*

Experiments. These were of two types.

Method 1. The culture was grown in the inorganic medium with varying amounts of galactose, from 2 to 10 %. The amount of the vitamin solution was reduced to one-third of the usual amount, with the object of limiting the growth of the cells but, it was hoped, not limiting the amount of galactozymase formed. After 36 hr., the cells were washed by centrifugation in the usual way, suspended in phosphate buffer and samples removed for the estimation of the enzyme. Counts and dry weight were also determined at the end of the growth period.

Results. In two experiments, there was equal growth in all concentrations of galactose. The amount of enzyme formed was in these instances found to be related to substrate concentration by a straight line passing almost through the origin (Fig. 5). The points in the figure refer to experimental results; the line represents the theoretical relationship and is calculated from Equation (1) and the experimental value at 10 % galactose. The value for K_m was 0·0038 M (see below).

In five other experiments, however, there was more growth in the solutions containing more galactose. Adaptation was still occurring in these at the end of the period of growth. It was consequently not possible to obtain reproducible results by this method, and a second type of experiment was adopted to achieve the necessary conditions to test this point.

Method 2. The cells were grown in the inorganic medium containing 5% glucose. They were centrifuged and washed as usual and then suspended in phosphate buffer at 29° containing varying amounts of galactose from 2 to 8%, together with 0·1% $(NH_4)_2SO_4$. At intervals, samples were removed for determination of galactozymase activity. The small amount of growth which occurred in these conditions was the same at all concentrations of galactose.

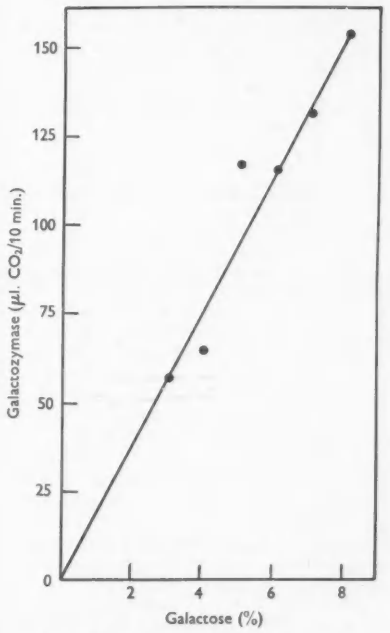

Fig. 6. Effect of galactose concentration on amount of galactozymase formed. No restriction of 'building blocks'. Yeast cells grown in glucose medium and suspended in buffer containing varying amounts of galactose, together with 0·1% $(NH_4)_2SO_4$. Points give experimental results; line calculated from theory (Equation 1) using experimental value found with 8% galactose.

Results. As in previous experiments, the rate of adaptation was initially independent of substrate concentrations. After 48 hr. of adaptation, however, equilibrium was established, resulting in a linear relationship between the amount of enzyme formed and the substrate concentration (Fig. 6). The line was calculated from Equation (1), using the experimental value at 8% galactose.

(b) Building blocks restricted

Experiments. Equation (2) involves K_m and K, both of which it is necessary to determine in order to calculate the relationship between the amount of enzyme and substrate concentration.

(i) *Determination of* K_m. Galactose-adapted cells were suspended in buffer solution containing galactose up to 0·024 M and CO_2 production measured at 38°. Four determinations were made and the mean value of K_m was 0·0038 M. Since K_m was determined in intact cells this value may be too high. However, in relation to the sugar concentrations used to produce adaptation (0·1–0·5 M) any such error in the determination is negligible.

(ii) *Determination of* K. The yeast was grown in the glucose medium, washed and placed in two flasks containing phosphate buffer with 2% (0·111 M) and 5% (0·278 M) galactose. At 12 hr. intervals the cells were centrifuged and resuspended in fresh solution to keep the sugar concentration constant. After 38–40 hr. the amount of enzyme produced in each of the solutions was measured. From these values (e_a and e_b), and knowing s_a and s_b, K was calculated from the equation

$$K=\frac{1}{e_b-e_a}\left(\frac{e_b s_b}{s_b+K_m}-\frac{e_a s_a}{s_a+K_m}\right). \qquad (3)$$

In one experiment e_a and e_b were respectively 17 and 35 for $s_a=0{\cdot}111$ M and $s_b=0{\cdot}278$ M. Substituting these values in (3)

$$K=\frac{1}{35-17}\left(\frac{35\times 0{\cdot}278}{0{\cdot}278+0{\cdot}004}-\frac{17\times 0{\cdot}111}{0{\cdot}111+0{\cdot}004}\right)$$
$$=1{\cdot}005.$$

The mean value of K obtained from 10 separate determinations was 1·006 ($\sigma=0{\cdot}001$).

(iii) *Determination of amounts of enzyme formed with varying concentrations of substrate: building blocks restricted.* A washed suspension was made from yeast grown in the glucose medium. Samples were set up with phosphate buffer containing 2–8% galactose and, after 40–48 hr., the amount of enzyme was measured. In earlier experiments the cells were transferred to fresh phosphate-galactose solution every 12 hr. Later experiments showed, however, that this was not necessary.

Results. The results of one experiment are shown in Fig. 7. These were further data obtained in the experiment shown in Fig. 4 and shown again in Fig. 7*A* as the degree of adaptation after 22 hr. Values at 30 and 48 hr. demonstrate that little further adaptation occurred with 3% galactose, but progressively increasing adaptation occurred with higher amounts of sugar. The shape of these two curves is similar, though the absolute values increased between 30 and 48 hr. Results of another experiment are shown in Fig. 8. Again, the points give the experimental results and the curve was calculated as in Fig. 7*B* and *C*.

It is of interest to compare Figs. 6 and 8, since the results were obtained from the same suspension of cells. The amount of enzyme formed in the absence

of ammonium sulphate is little more than half of the amount formed in the presence of the salt.

Conclusion. The effect of restricting the supply of building blocks is twofold. The first is to reduce the total amount of enzyme formed. The second is to alter the relationship between substrate concentration and amount of enzyme formed from a straight line to one in which diminishing amounts of enzyme are produced by progressive increments of substrate. These findings are qualitatively and quantitatively in accordance with the theoretical predictions.

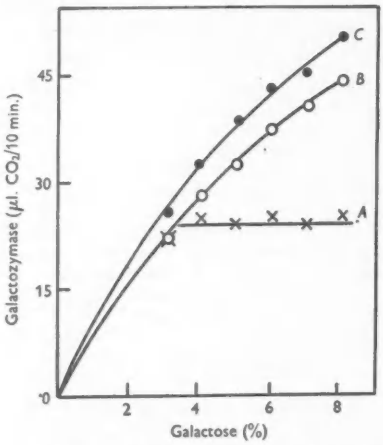

Fig. 7. Effect of galactose concentration on amount of galactozymase formed. 'Building blocks' restricted. Yeast cells grown in glucose medium and suspended in buffer containing varying amounts of galactose. Curve *A*, 22 hr. adaptation. Initial rate of enzyme production is independent of substrate concentration (see Fig. 4). Curves *B* and *C*, 30 and 48 hr. adaptation. Amount of enzyme formed proportional to substrate concentration; points represent experimental results, curves calculated from theory (Equation 2), using experimental values found with 8% galactose.

The relative amounts of galactozymase and maltozymase formed in the presence of both substrates

Let us consider the situation in which cells are suspended in a solution containing two 'adaptive' substrates and in which the respective enzymes are in equilibrium with a common restricted pool of building material and hence in equilibrium with each other. Both enzymes will be produced, probably at a different rate. That which is produced faster will tend to take a greater share of building material and so restrict this for the other enzyme. This second enzyme should, therefore, at this stage increase slowly or not at all. If the substrates are not replenished, the first enzyme will reach a maximum, either because it has come into equilibrium with the existing concentration of substrate or because the substrate is completely exhausted. In either case, because of the continued fall in substrate concentration or its absence, there will be a decrease in the amount of the enzyme, so liberating material for the building of the second enzyme. Thus the second enzyme should now increase at the expense of the first. Apart therefore from the earliest stages of simultaneous adaptation, there will be a continuously changing ratio of the two enzyme concentrations.

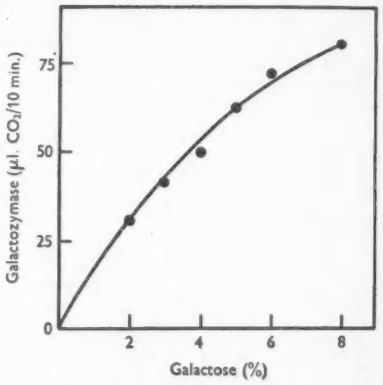

Fig. 8. Effect of galactose concentration on amount of galactozymase formed. 'Building blocks' restricted. Yeast cells grown in glucose medium and suspended in buffer containing varying amounts of galactose. Effect of presence or absence of building blocks shown by comparing Figs. 6 and 8, since same suspension of cells used in both experiments. Points give experimental results; curve calculated from theory (Equation 2) using experimental value found with 8% galactose.

If the concentrations of the substrates are held constant, the ratio of the enzymes will tend towards a constant value given by

$$\frac{e_1}{e_2}=\frac{K_{e_1}}{K_{e_2}}\frac{K_{m_2}}{K_{m_1}}\left(\frac{s_1+K_{m_1}}{s_2+K_{m_2}}\right), \qquad (4)$$

where e_1 and e_2 are the concentrations of the two enzymes with Michaelis constants of K_{m_1} and K_{m_2}, s_1 and s_2 are the substrate concentrations and K_{e_1} and K_{e_2} are the equilibrium constants for the reactions $B \rightleftharpoons E_1$ and $B \rightleftharpoons E_2$.

The conditions under which this equation is strictly valid are in general unobtainable since, in the derivation of the equation, it is assumed that both enzymes are in an equilibrium state simultaneously. The equation can, therefore, in most instances, have only semi-quantitative application. It is nevertheless of interest to compare the theoretical prediction

with the experimental results. For this it is necessary to know K_{m_1}, K_{m_2}, K_{e_1} and K_{e_2}.

The Michaelis constant for galactozymase has already been determined, $K_{m_1} = 0{\cdot}0038$ M. For maltozymase, K_{m_2} was determined in the same way and its value is 0·0028 M.

K_e can be obtained from K (Equation 3), since $K_e = K - 1$. For galactozymase, K was found to be 1·006, whence $K_{e_1} = 0{\cdot}006$. For maltozymase, $K_{e_2} = 0{\cdot}003$. Both of these values, and especially that for maltozymase, can only be taken as rough approximations, since they depend on the fourth significant figure in the calculated value of K. Thus, absolute values for e_1/e_2 can be obtained only very approximately. However, it should be possible to calculate changes in the value of e_1/e_2 with changes in s_1 and s_2 with some degree of accuracy. When s_1 and s_2 are varied, the variation of e_1/e_2 is independent of the constant term $(K_{e_1} K_{m_2})/(K_{e_2} K_{m_1})$, and e_1/e_2 is directly proportional to $(s_1 + K_{m_1})/(s_2 + K_{m_2})$.

Experiment 1. This experiment was designed to study the fluctuation of the two enzymes in solutions in which the substrates were not renewed. Cells grown in the glucose medium were washed and placed in two flasks with different concentrations of galactose and maltose. The first contained galactose 1·4% (0·078 M) and maltose 3·6% (0·100 M); the second contained galactose 3·6% (0·200 M) and maltose 1·4% (0·039 M).

Results. Fig. 9 shows how the amounts of the two enzymes change between 10 and 38 hr. adaptation. Other noteworthy features are the more rapid formation of galactozymase in both solutions, the retardation of maltozymase formation when its relative concentration was lower, i.e. in the second solution, and the rise in maltozymase in both solutions associated with the fall of galactozymase. Similar results have been obtained by Spiegelman & Dunn (1947).

Experiment 2. Cells grown in glucose medium were washed and suspended in two flasks containing buffer solutions with the following amounts of galactose and maltose: (1) galactose 1·4% (0·078 M), maltose 3·6% (0·100 M); (2) galactose 3·6% (0·200 M), maltose 1·4% (0·039 M).

The suspensions were kept at 29° and the cells transferred to fresh solutions every 12 hr. At 40 hr., samples were removed and washed and the activity of the enzymes determined.

Results. A relative increase in concentration of galactose to maltose of about sixfold (s_1/s_2 from 0·78 to 5·1) should give about the same relative increase in the ratio of the enzymes (Table 1). In fact, the relative increase in the enzymes was about twofold.

This degree of discrepancy was not unexpected since, as we have seen, theoretical values can only be achieved in conditions where both enzymes are in equilibrium with their substrates at about the same time.

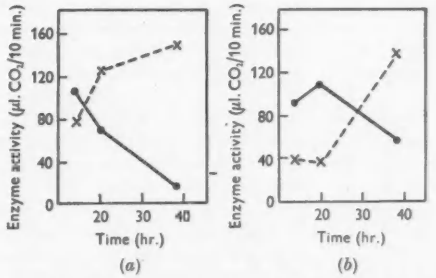

Fig. 9. Simultaneous production of galactozymase and maltozymase; competition in enzyme formation. ●—●, Galactozymase; × --- ×, maltozymase. Yeast cells grown in glucose medium and suspended in solutions containing (*a*) galactose 0·078 M, maltose 0·100 M; (*b*) galactose 0·200 M, maltose 0·039 M. Solutions not renewed during adaptation.

Experiment 3. This experiment was carried out in a similar way to Exp. 2, but with two differences designed to give conditions nearer to those necessary for simultaneous equilibrium of the two enzymes (see Mandelstam, 1952). Firstly, the concentrations of the substrates were lower: (1) galactose 1·6% (0·089 M), maltose 0·53% (0·015 M); (2) galactose 0·53% (0·030 M), maltose 1·6% (0·0445 M). Secondly, the cells were suspended in twice the usual volume of sugar solutions, so that the concentration of cells was about 15 mg. dry wt./ml.

Results. As expected, agreement between theoretical and experimental values was better than in the previous experiments (Table 1). The calculated

Table 1. *Effect of variation in substrate concentrations on formation of galactozymase and maltozymase*

(Enzyme concentration expressed as μl. CO_2 liberated in 10 min.)

Exp. no.	Suspension	Substrate concn. (M) Galactose s_1	Maltose s_2	s_1/s_2	Enzyme concn. Galactozymase e_1	Maltozymase e_2	e_1/e_2	R_1/R_2 Found	Calculated
2	1	0·200	0·039	5·1	115	25	4·6 (R_1)	2·1	5·9
	2	0·078	0·100	0·78	87	40	2·2 (R_2)		
3	1	0·089	0·015	5·9	84	34	2·5 (R_1)	6·2	7·1
	2	0·030	0·044	0·67	44	109	0·4 (R_2)		

increase in the ratio of e_1/e_2 was about sevenfold; the increase found was about sixfold.

Experiment 4. In this experiment, the conditions were as in Exp. 3, except that the concentration of maltose was held constant and that of galactose increased. Four flasks were set up, each with maltose 0·53% (0·015M) and with galactose 0·53% (0·030M), 1·06% (0·059M), 1·6% (0·089M) and 2·13% (0·119M); the enzymes were measured after 44 hr. of adaptation.

Results. The results are given as the points in Fig. 10, and the straight line represents the theoretical results, calculated from Equation (4), and from the experimental value found with galactose at 0·030M.

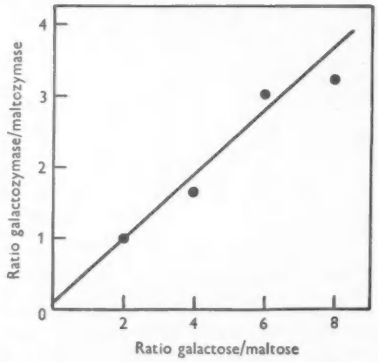

Fig. 10. Effect of relative concentrations of galactose and maltose on production of the two enzymes. Yeast grown in glucose medium and suspended in solutions containing the two sugars; the solutions were renewed every 12 hr. Maltose held constant (0·015M) and galactose increasing (0·030, 0·059, 0·089 and 0·119M) produces linear increase in the ratio of the two enzymes. Points give experimental results; line calculated from theory (Equation 4), using value found with galactose 0·030M.

Conclusion. In the introduction to this section, it was explained that quantitative predictions from the theory concerning simultaneous adaptation to two substrates are more difficult to verify than other predictions in which only one enzyme is involved. Nevertheless, the experimental results which we have obtained conform well to the predictions made.

DISCUSSION

The experiments have confirmed all the predictions from the theory which have been tested. We may now refer to investigations reported by other workers which support our own findings. Firstly, we have shown that the rate of adaptation is sometimes linear, but is sometimes of an exponentially increasing type. Both types of curve are to be expected theoretically, the former when there is no increase in material to the pool of building blocks and the latter when there is such an increase. Knox (1951) has also demonstrated two types of curve with the production of tetrathionase by *Salmonella paratyphi* B. With tetrathionate alone, the rate of production of the enzyme was linear. With the addition of mannitol or glucose, there was an increasing rate of production of the enzyme, though it is not clear whether the increase was exponential or not.

We ourselves have shown that a linear rate of adaptation occurs with hepatic arginase of adult rats, where we can assume negligible increase of material in the pool of building blocks (Mandelstam & Yudkin, 1952).

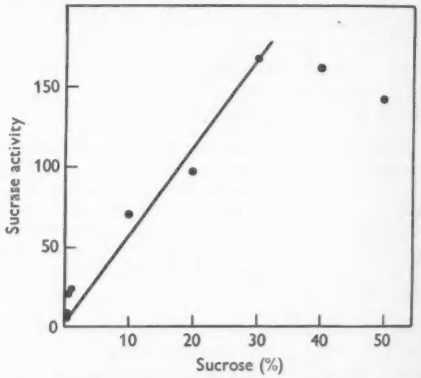

Fig. 11. Effect of sucrose concentration on production of sucrase by *Penicillium glaucum*. Points give experimental results found by Kertész (1928); line calculated from theory (Equation 1), using experimental value found with sucrose 30%. The lower amounts of enzyme found with concentrations above 30% are attributable to the injurious effect of these high amounts of sugar upon the organisms.

Secondly, in conditions where varying amounts of substrate did not affect the growth of the culture, the amount of enzyme produced was found by us to be directly proportional to the concentration of substrate. An experiment reported by Kertész (1928) produced similar results. He grew cultures of *Penicillium glaucum* in media containing from 0·01 to 50% of sucrose, and after 12 days estimated the amount of sucrase formed. Kertész says that considerable growth had occurred after 8 days, so that it is likely that little further growth was occurring after 12 days. We should then expect, as in our own experiments, that the amount of sucrase formed would increase linearly with increase in sucrose. We have plotted in Fig. 11 the results of Kertész, together with the theoretical curve calculated from Equation 1, using his value of sucrase for 30% sucrose. Kertész concluded that there was a linear relationship between the amount of enzyme formed

and concentration of sucrose up to 30 % sugar, above which the high concentrations of sucrose have an adverse effect upon the organism. The figure shows that the theoretical curve agrees quite well with the experimental values up to 30 % sugar.

Thirdly, the experiments concerning simultaneous adaptation to two substrates recall the work of Monod (1945). In experiments with *Escherichia coli*, he has studied the rate of growth in the presence of the 'constitutive' substrate glucose and one of a series of 'adaptive' substrates such as xylose. It may be taken that the rate of growth is proportional to the amount of the two enzymes present. With similar concentrations of the two sugars, growth proceeded until the glucose was exhausted and then there was a pause in the growth whilst adaptation to xylose was taking place. Monod showed that this pause decreased progressively with an increase in the ratio of xylose to glucose. When this ratio was 1000 to 1, growth proceeded without a pause, indicating that in these conditions adaptation to xylose was occurring whilst glucose was still present. Thus, adaptation can be induced in the continuing presence of a 'constitutive' substrate by making the relative concentration of 'adaptive' substrate sufficiently high.

These results are in conformity with the 'mass action' theory. Monod, like ourselves, assumes that the distinction between a constitutive enzyme and an adaptive enzyme is quantitative rather than qualitative and depends upon whether the equilibrium between precursor and enzyme lies more toward the latter or the former. The theory then predicts that adaptation in the presence of both a 'constitutive' and an 'adaptive' substrate will occur, as Monod has shown, when the concentration of the 'adaptive' substrate is relatively very high. Adaptation in the presence of two 'adaptive' substrates, on the other hand, will occur, as we have shown, when the two concentrations are of the same order.

SUMMARY

1. Predictions made from the 'mass action' theory of enzyme adaptation have been tested in experiments on the adaptation of yeast cells to the fermentation of galactose.

2. The rate of adaptation in a given concentration of galactose was found to be either linear or exponentially increasing. In no instance was a linear rate of adaptation found when there was an increase in cell mass during adaptation.

3. The initial rate of adaptation was independent of the concentration of galactose over the tested range of 3–8 %.

4. When there was no restriction of material for the building of the enzyme, the amount of galactozymase formed was linearly related to the concentration of galactose. When there was such a restriction, there was a decreasing increment of enzyme with increasing concentration and the total amount of enzyme formed was less.

5. The relative amounts of galactozymase and maltozymase formed in the presence of both sugars are determined by the relative amounts of galactose and maltose present.

6. These results, and some others reported in the literature, are quantitatively in agreement with the predictions made from the theory.

We acknowledge with gratitude financial assistance from Messrs Allen and Hanburys, The British Drug Houses and H. W. Carter and Co. Ltd. We are also grateful to Miss Joan Fleming for technical assistance. One of us (J.M.) is indebted to the Medical Research Council for a personal grant.

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Steroids of Pregnant Mares' Urine

4. FRACTIONATION OF THE NEUTRAL STEROIDS. EXAMINATION OF SOME NON-KETONIC FRACTIONS

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Previous papers in this series (Klyne, Schachter & Marrian, 1948; Klyne, 1948; Paterson & Klyne, 1948) have described the isolation from pregnant mares' urine of 5α-pregn-16-en-3β-ol-20-one, uranediol and 5α-pregnane-3β-ol-20-one as their sulphuric acid esters. This paper describes the fractionation of the neutral steroids of pregnant mares' urine and the detailed examination of some non-ketonic fractions. Most previous workers have concentrated their attention on the ketonic fractions (see, for example, Heard & McKay, 1939; Heard & Hofmann, 1940, 1941; Oppenauer, 1941; Prelog & Führer, 1945; the last-mentioned paper gives full references to earlier work). Marker and his colleagues in the years 1937–9 (see references on p. 707) dealt extensively with both ketonic and non-ketonic fractions, but their work was carried out with commercial urine extracts which had been so harshly treated that some of the compounds isolated may have been artifacts. Bauld & Heard (1940) have carried out a brief examination of the non-ketonic steroid fraction.

Preliminary accounts of parts of the present work have already been given (Klyne & Paterson, 1948; Brooks, Klyne & Miller, 1951*a*). The identification of uranediol as a *D*-homosteroid has been described elsewhere (Klyne, 1950).

Nomenclature follows the rules proposed by a conference at the CIBA Foundation, London, and published in *J. chem. Soc.* (1951), p. 3526. Steroid rings are distinguished as *A*, *B*, *C* and *D* (italic capital letters) and not A, B, C and D (Roman small capitals) as shown in these rules. Small capitals are undesirable since D (and L) are used for another purpose in designating configurations relative to glyceraldehyde or serine (cf. *Biochem. J.* 1948, **42**, 1). Prof. T. Reichstein draws attention to this point in the German version of the steroid nomenclature rules (*Helv. chim. Acta*, 1951, **34**, 1680).

Separation of the major steroid fractions

The extraction of the steroid conjugates from pregnant mares' urine and their fractionation have been described by Klyne *et al.* (1948). The material used for the present study consisted of the conjugates in the 'water-insoluble' and 'water-soluble' fractions which could not be purified as sulphates. These conjugates were hydrolysed to the free steroids by hydrolysis with N-hydrochloric acid at 100°, and the free steroids were separated into phenolic and non-phenolic fractions by extraction of their ether and benzene solutions with sodium hydroxide. The major separations are outlined in Fig. 1. In the course of these separations on one large batch of extracts, a small neutral fraction insoluble in benzene was removed. This proved to consist largely of trihydroxysteroids and was called the 'Triol Fraction'.

The non-phenolic material was separated into ketonic and non-ketonic fractions by the reagent T of Girard & Sandulesco (1936), and these fractions again into digitonin-precipitable (β) and digitonin-non-precipitable (α) fractions. For one batch the digitonin precipitation was carried out in 90% (v/v) methanol according to Shoppee (1946); for another batch it was carried out in 95% (v/v) ethanol at room temperature followed by precipitation of the more soluble digitonides with ether as suggested by Marker, Rohrmann & Wittle (1938). The latter method proved a simple way of obtaining uranediol from the urine extracts.

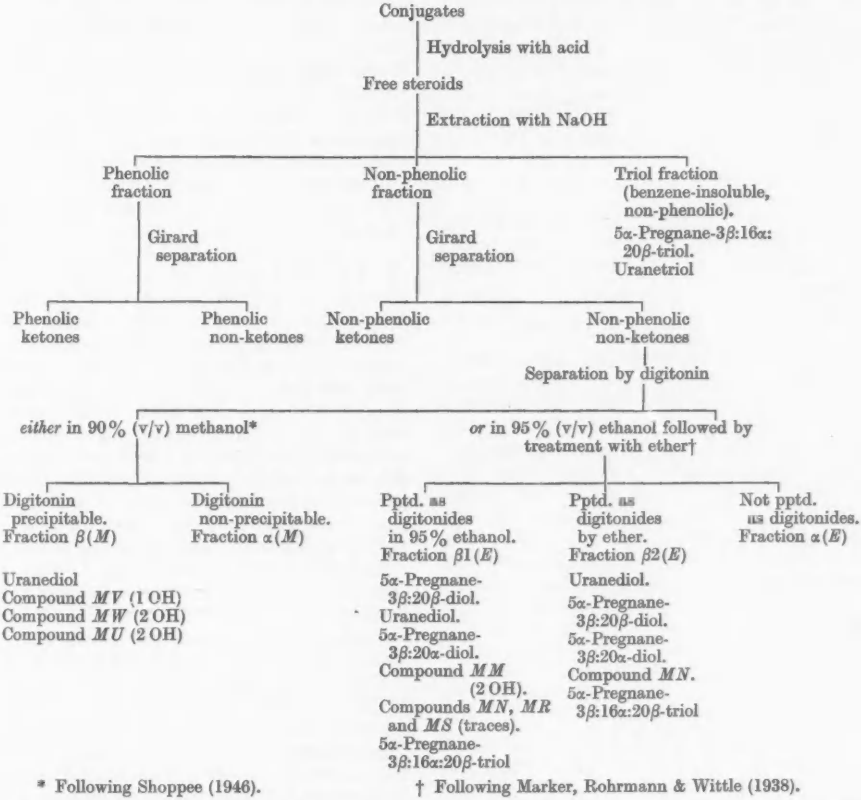
The non-ketonic β fractions (β(*M*), β1(*E*) and β2(*E*) in Fig. 1) were then submitted to chromatography on alumina as free alcohols, as acetates and as benzoates.

The non-ketonic β fractions

Separation by digitonin in methanol

The non-phenolic, non-ketonic fraction from 530 l. urine (2·39 g.) was submitted to separation by digitonin in methanol following Shoppee (1946), and the β(*M*) fraction was chromatographed over alumina. Benzene eluted a little material which apparently consisted of monohydroxy compounds; an imperfectly characterized compound *MV*, m.p. 61–63°, was obtained from this fraction. Most of the material was eluted with benzene-ether mixtures. This fraction (*B*) was acetylated and the acetates

were chromatographed over alumina. Three main groups of fractions (*BA*, *BB*, *BC*) were eluted with hexane-benzene mixtures. The second group (*BB*) yielded after tedious fractional crystallizations and chromatography small quantities of uranediol diacetate, and of a new acetate called provisionally unsaturated to tetranitromethane and shows absorption in the ultraviolet at about 200 mμ., which is compatible with the presence of an olefinic linkage (cf. Bladon, Henbest & Wood, 1951; Halsall, 1951). Its melting point is higher than that of any known isomer of pregnane- or pregnene-diol diacetate.



* Following Shoppee (1946).

† Following Marker, Rohrmann & Wittle (1938).

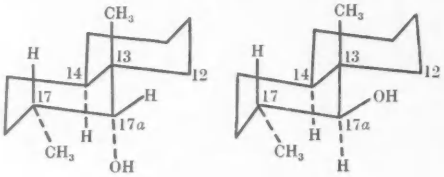
Fig. 1. Separation of the major fractions from mares' urine extracts. The compounds so far isolated in this investigation are listed under the various fractions. The only other non-phenolic, non-ketonic 3β-hydroxysteroid isolated from pregnant mares' urine is pregn-5-ene-3β:20α-diol (Marker & Rohrmann, 1938). Marker & Rohrmann (1939) obtained evidence of the presence of a urenetriol and a new 5α-pregnanetriol.

compound '*MU* diacetate', m.p. 206–208°. The many side-fractions obtained in this process were later pooled and treated by the digitonin process of Marker (see p. 696). The third group of fractions (*BC*) after repeated crystallizations yielded a further new compound '*MW* diacetate', m.p. 175·5–178°.

Compound MU. The analysis of the acetate of this compound and its behaviour on chromatography suggest that it is a diacetate $C_{25}H_{38-40}O_4$; it is

Compound MW. The analysis and chromatographic behaviour of *MW* acetate suggest the formula $C_{25}H_{40}O_4$; it is saturated to tetranitromethane. Vigorous hydrolysis of the diacetate with potassium hydroxide in methanol yielded uranediol: but *MW* diacetate ($[M]_D$, −218) could not be a polymorphic modification of uranediol diacetate ($[M]_D$, −120). Lack of material has prevented a further study of *MW* diacetate, but it seems possible that compound *MW* may be the 17*a*-epimer of

uranediol, in which the hydroxyl group at position 17*a* has the less stable 'polar' 17*a* α-position (as in I). On vigorous treatment with alkali this might perhaps rearrange to give the more stable 'equatorial' 17*aβ*-hydroxyl group (as in II). (See the general discussion of 'polar' and 'equatorial' bonds



I II

in steroids by Barton, 1950, where many examples of the transformation of 'polar' to 'equatorial' hydroxyl groups under the influence of alkali are described.)

Evidence for 17aβ configuration of uranediol

The formulation of uranediol as a 17α-methyl-*D*-homoandrostane-3*β*:17*a*-diol has been discussed by one of us previously (Klyne, 1950). The molecular rotation of uranediol dibenzoate, which has now been determined, shows that the 17*a*-hydroxyl group in this compound is *β*-oriented. The indices α and *β* at $C_{(17a)}$ are allotted here in their modern significance according to the proposals made by Goldberg, Sicé, Robert & Plattner (1947) on the basis of the rates of hydrolysis of the 17*a* esters and the androgenic activity of the alcohols. Goldberg *et al.* in their paper of 1947 actually use the 'old' indices ('α' for the androgenically active compounds which are now referred to as *β*-compounds).

The following are the calculations supporting the conclusion that uranediol is a 17*aβ*-compound.

$[M]_D$ Uranediol dibenzoate (+118)
$-[M]_D$ uranediol (+13)
= increment on benzoylation
(Δ_2 value at $C_{(3)}$ and $C_{(17a)}$)
= +105.

The Δ_2 value at $C_{(3)}$ for a 3*β*-hydroxy-5α-steroid (Barton & Cox, 1948) is +5. Therefore Δ_2 value at $C_{(17a)}$ = +100 for uranediol.

For 17*a*-epimeric alcohols Goldberg *et al.* (1947) found the following Δ_2 values: at 17*aα*, −152; 17*aβ*, +227. Exact agreement between these Δ_2 values and that for 17*a* in uranediol dibenzoate cannot be expected because of the possibility of vicinal action between the two benzoate groups, and between the $C_{(17a)}$ benzoate group and the $C_{(17)}$ methyl. However, the positive sign of the Δ_2 value in uranediol dibenzoate indicates that it most probably has the 17*aβ*-configuration.

The compounds *MM*, *MV* and *MW* were named *M*, *V* and *W* in our preliminary communications (Klyne & Paterson, 1948; Brooks *et al.* 1951*a*). They have been renamed with the prefix *M* to avoid confusion with the adrenal steroids of Reichstein.

Separation by digitonin in ethanol and ether

The non-ketonic material from 270 l. urine (5·18 g.) treated according to Marker, Rohrmann & Wittle (1938) yielded the following three fractions: *β*1(*E*), compounds precipitated by digitonin in 95% (v/v) ethanol at 15–20° (1·03 g.); *β*2(*E*), compounds not precipitated by digitonin in 95% ethanol, but precipitated in ether (380 mg.); α(*E*), compounds not precipitated by digitonin (3·5 g.).

*Fraction β*1(*E*). This material was chromatographed as free alcohols on alumina, yielding three main fractions eluted with benzene-ether mixtures, I*A*, I*B*, and I*C* (total about 800 mg.); the less easily eluted fractions (about 200 mg.) have not been studied in detail. Each of the three main fractions was again chromatographed; I*A* gave a gum, very easily eluted with benzene containing 10% ether, and then some impure uranediol: I*B* gave more impure uranediol and 5α-pregnane-3*β*:20*β*-diol (cf. Bauld & Heard, 1940): I*C* (410 mg.) gave much material which after crystallization from ethyl acetate-light petroleum appeared to be fairly pure 5α-pregnane-3*β*:20*β*-diol (170 mg.). Data proving the identity of this compound (m.p. 194·5–196°) with authentic material are given in Table 2 (p. 702).

Chromatography of benzoates. The finding of 5α-pregnane-3*β*:20*β*-diol in such large quantities led to a search for the 20α-epimer, which had been isolated from pregnant mares' urine by Marker & Rohrmann (1938). It was known from previous synthetic work (cf. Klyne & Barton, 1949) that 20-epimeric hydroxysteroids could not be readily separated by chromatography of their acetates. It was, therefore, proposed to try the use of the benzoates, which had been employed successfully by Barton & Miller (1950) to separate the epimeric 5α:6α- and 5*β*:6*β*-epoxycholestan-3*β*-ols. Pilot experiments with synthetic mixtures of 20-epimeric benzoates showed at once that these could be separated almost quantitatively in a single chromatogram on alumina, the 20*β*-benzoate being eluted before the 20α. (These pilot experiments have been described briefly by Brooks, Klyne & Miller, 1951*b*; a fuller account of this work is in preparation. Turner & Voitle (1951) described the use of benzoates for separating 20-epimeric steroids almost simultaneously with us.)

All those portions of fraction *β*1(*E*) which had not been separated as pure uranediol or 5α-pregnane-3*β*:20*β*-diol were benzoylated and chromatographed. The chromatograms yielded four major constituents

as dibenzoates in the following order: 5α-pregnane-3β:20β-diol, 5α-pregnane-3β:20α-diol, uranediol and a new *compound MM*. All these benzoates have sharp melting points and crystallize well; a solvent suitable for crystallizing many steroid benzoates is chloroform-methanol (20:80 v/v). This appears to be the first occasion in which a pair of 20-epimeric hydroxysteroids has been isolated from the same urine. Data proving the identity of the 5α-pregnane-3β:20α-diol from mares' urine with authentic material are given in Table 4. The constitution of compound *MM* has not been elucidated. From the analysis of its benzoate and from the chromatographic behaviour of the alcohol and the benzoate it seems probable that it is an isomer of pregnanediol ($C_{21}H_{36}O_2$) or pregnenediol ($C_{21}H_{34}O_2$). It is not identical with pregnane-3β:20β-diol or 5α-pregn-16-ene-3β:20β-diol, which it resembles in some properties (see Table 5).

Fraction β2(*E*). This was chromatographed on alumina after the removal of material insoluble in benzene. Most of the material was eluted with benzene-ether mixtures; the earlier fractions yielded almost pure uranediol, the later fractions, crude 5α-pregnane-3β:20β-diol. As was the case with fraction β1(*E*) the compounds could be separated much more easily as benzoates than as alcohols. Thus uranediol and 5α-pregnane-3β:20β-diol appeared in both β1 and β2 fractions; the 5α-pregnanediol chiefly in the β1 fraction and the uranediol in the β2 fraction.

The benzene-insoluble portion of fraction β2(*E*) was chromatographed after benzoylation. 5α-Pregnane-3β:20β-diol dibenzoate was eluted first, followed by a fraction consisting of the tribenzoates of the triols—chiefly 5α-pregnane-3β:16α:20β-triol.

Quantities isolated. It is impossible after such a lengthy fractionation process to estimate the quantities of the various steroids originally present in the urine, but the quantities of the principal steroid diols isolated in a fair state of purity from 270 l. urine are as follows. (The figures include material isolated as alcohols, as benzoates and as sulphate, all calculated as alcohols.) Uranediol, 390 mg.; 5α-pregnane-3β:20β-diol, 250 mg.; 5α-pregnane-3β:20α-diol, 20 mg.; compound *MM*, 40 mg.

Fraction α(*E*). This has not been investigated fully, but the benzene-insoluble portion of it yielded a new polymorphic modification of uranetriol triacetate.

The triol fraction

This fraction contained no phenols and very little ketonic material. The non-ketonic portion has yielded two compounds, namely 5α-pregnane-3β:16α:20β-triol (first isolated by Haslewood, Marrian & Smith, 1934; constitution determined by Hirschmann, Hirschmann & Daus, 1949) and uranetriol (Marker, Kamm, Crooks, Oakwood, Wittle & Lawson, 1938; Marker, Kamm, Oakwood, Wittle & Lawson, 1938). No generally satisfactory method of separating these triols has yet been found. The two isomers could be separated from one batch of extract by fractional crystallization of their acetates from methanol and aqueous methanol (Marker, Kamm, Crooks *et al.* 1938). The identity of our uranetriol triacetate (m.p. 135–136°) and that of Marker (m.p. 136°) was proved by the identity of their infrared absorption spectra which were kindly determined by Dr K. Dobriner and Mrs P. Humphries of the Sloan-Kettering Institute, New York. The triol fraction from the other batch of extract could not be separated by chromatography and fractional crystallization of the acetates in this way. However, fractional crystallization of the mixed acetates from light petroleum followed by methanol yielded a compound of m.p. 153–154°; this was shown by the identity of its infrared absorption in carbon disulphide with that of uranetriol triacetate (m.p. 135–136°) to be a polymorphic modification of the latter. Both forms of uranetriol triacetate on alkaline hydrolysis gave uranetriol, the melting point of which agreed with that found by Marker, Kamm, Crooks *et al.* (1938).

After it had been found that steroid diols, which could not be separated efficiently by chromatography of their acetates, could often be separated well by chromatography of their benzoates, attempts were made to separate the two triols in this way. These attempts were unsuccessful except where one or other triol predominated in the mixture; in such cases the predominant isomer could be obtained pure.

EXPERIMENTAL

Material

The material used consisted of all the 'water-insoluble' conjugate fractions (Klyne *et al.* 1948), which had not been separated as sulphates of 5α-pregn-16-en-3β-ol-20-one, 5α-pregnane-3β-ol-20-one and uranediol, and the 'water-soluble' conjugate fractions from two batches of pooled late pregnancy urine. Batch *B* (270 l. urine) was obtained from the Ovaltine Research Laboratories and British Drug Houses Ltd.; batch *D* (530 l. urine) was obtained from N.V. Organon, Oss, Netherlands.

General methods

Melting points. These were determined on the hot-stage apparatus of Klyne & Rankeillor (1947), and are corrected for emergent stem.

Specific rotations. These were determined for the *D* line, using a 0·5 dm. microtube. The errors are calculated as described by Klyne *et al.* (1948) except that five pairs of readings with the solutions and five pairs with a solvent blank were taken in each case. Temperature, where not stated, was 20–25°.

Micro-analyses. These are by Dr J. W. Minnis, Edinburgh, or Drs Weiler and Strauss, Oxford. Compounds were dried to constant weight at 110° *in vacuo* unless stated otherwise.

Ultraviolet absorption spectra. These were determined in ethanol (sometimes containing 1% (v/v) $CHCl_3$), using a Beckman spectrophotometer, model DU.

'*Usual working-up.*' See Klyne (1948).

Chromatograms. These were carried out using Al_2O_3 as described by Paterson & Klyne (1948). The proportions of mixed solvents are given as % (v/v). The activity of the Al_2O_3 was determined according to Brockmann & Schodder (1941), and was II–III where not stated otherwise.

Light petroleum was the fraction of b.p. 60–80°.

Benzoates. These were prepared by dissolving the steroid in pyridine (1 ml./100 mg.), cooling the solution in ice and adding benzoyl chloride dropwise (0·1 ml./100 mg. of steroid for each hydroxyl group). The solution was allowed to stand for 18 hr. at room temperature and then ice was added to react with the excess benzoyl chloride. After being allowed to stand for a further 2–3 hr. the mixture was worked up in the usual way.

Acetates. These were prepared in the same way as the benzoates using acetic anhydride (0·2 ml./100 mg. of steroid) in place of benzoyl chloride.

Hydrolysis of conjugates

The following notes describe the hydrolysis and fractionation of batch *D*. Batch *B* was treated in a similar way, except for the digitonin separation described on p. 701. The 'water-insoluble' and 'water-soluble' fractions were combined in 2·5 l. water; conc. HCl (200 ml., 11 N) was added and the mixture was heated at 100° for 2 hr. It was cooled and extracted with ether (2 × 500 ml., 1 × 300 ml., 3 × 200 ml.); a little tarry material which adhered to the sides of the separating funnels was dissolved in ethanol and added to the ether extracts. The aqueous solution was rejected and the ethereal extracts used for the separation of phenols from non-phenols.

Separation of the major steroid fractions

Phenolic and non-phenolic fractions. This separation is shown diagrammatically in Fig. 2.

Since weak phenols cannot be readily extracted with N-NaOH from ether, but can be extracted by this reagent from benzene, the 'non-phenolic' fraction I from the ether was transferred to benzene and then was further extracted. This method was very clumsy, due to the unexpected appearance of a benzene-insoluble fraction (later called the triol fraction). In future we propose to extract the original hydrolysis mixtures with benzene, filter off the benzene-insoluble material, and extract the benzene solutions with N-NaOH.

The products finally obtained were: triol fraction, 0·7 g. pale-buff solid; non-phenolic fraction II, 5·54 g. dark-brown gum; phenolic fractions (I and II), 1·50 g. pinkish sticky crystalline solid. The triol fraction gave a negative Millon reaction, and therefore contained little or no phenolic material.

Non-phenolic ketones and non-ketones. The non-phenolic fraction was separated further using the hydrazide reagent T of Girard & Sandulesco (1936) in the conditions described by those authors. The first Girard separation yielded a ketonic fraction (2·25 g.) and a non-ketonic fraction (2·82 g.). Each of these fractions was submitted to a further Girard treatment since this process does not effect a clean-cut separation. The final ketonic fraction weighed 1·59 g. and the final non-ketonic fraction 2·39 g.

Separation of non-phenolic non-ketones by digitonin in methanol

The final non-ketonic fraction from the Girard separation of batch *D* was submitted to digitonin separation in 90% methanol as described by Shoppee (1946) yielding the following fractions: non-ketonic, precipitated by digitonin; fraction β (*M*), 968 mg.; non-ketonic, not precipitated by digitonin, fraction α(*M*), 843 mg.

Fraction β(M)

This fraction (968 mg.) was dissolved in benzene and chromatographed on 30 g. Al_2O_3 (activity I–II) giving the following fractions (solvents used for elution are given in parentheses). *A* (benzene), 30 mg.; *B* (benzene-ether mixtures), 727 mg.: *C* (ether), 15 mg.; *D* (acetone), 42 mg.; *E* (methanol), 39 mg.

The main fraction *B* from the first chromatogram was acetylated and the acetates dissolved in 5 ml. benzene and 95 ml. hexane and chromatographed on Al_2O_3 (27 g., activity IV). The following fractions were obtained: *BA* (hexane:benzene, 95:5), 253 mg. gum; *BB* (hexane:benzene, 95:5), 340 mg. crystalline solid; *BC* (hexane:benzene, 50:50 and 40:60), 169 mg. crystalline solid; *BD* (benzene), 33 mg. partly crystalline; *BE* (ether), 62 mg. gum.

Compound MV. Fraction *A* was again chromatographed on 1 g. Al_2O_3 (activity IV). Pentane eluted 27 mg. partly crystalline material, which when crystallized twice from methanol-ether formed leaflets, m.p. 61–63°. This substance, provisionally called *compound MV*, was readily soluble in ether or $CHCl_3$ and sparingly soluble in methanol. It gave a precipitate with digitonin in 90% (v/v) ethanol. It gave no colour with tetranitromethane in $CHCl_3$, or in the Liebermann-Burchard test. It was sublimed for analysis at 90–115°/3 μ. (Found, C, 84·1; H, 13·7. $C_{21}H_{36}O$ requires C, 82·9; H, 11·9. $C_{27}H_{48}O$ requires C, 83·5; H, 12·4. $C_{30}H_{56}O$ requires C, 84·6; H, 11·7. $C_{39}H_{72}O$ requires C, 84·2; H, 12·0%.) Lack of material prevented further work on this compound.

Compound MU diacetate. Fraction *BB* was crystallized from hexane (4 ml.) giving 66 mg. of crystals (fraction *BB*1), m.p. 150–165°. This material after three further crystallizations from hexane yielded 7·5 mg. of thick plates, m.p. 202–206° after subliming at about 180° and softening at 197°. This material, provisionally called *compound MU diacetate* was sublimed at 160–180/1 μ. (Found C, 74·4; H, 9·1. $C_{25}H_{38}O_4$ requires C, 74·6; H, 9·5. $C_{25}H_{40}O_4$ requires C, 74·2; H, 10·0%.) $[\alpha]_D^{22°}$ − 89·5 ± 1·0° in $CHCl_3$ (*c*, 0·58). If molecular formula is $C_{25}H_{38}O_4$, $[M]_D$ is −360.

The ultraviolet absorption of compound *MU* diacetate in the region 200–220 mμ. was measured with a Unicam SP 500 spectrophotometer (at Birkbeck College, University of London, through the courtesy of Dr D. H. R. Barton). The compound showed the following values for ϵ (assuming the molecular formula $C_{25}H_{38}O_4$): at $\lambda_{max.}$, 203 mμ.; ϵ, 2240: at 210 mμ.; ϵ, 810: at 220 mμ.; ϵ, 87. These values are compatible with the presence of an olefinic linkage, probably trisubstituted (cf. Bladon *et al.* 1951; Halsall, 1951). The compound gave a faint but definite yellow colour with tetranitromethane in $CHCl_3$.

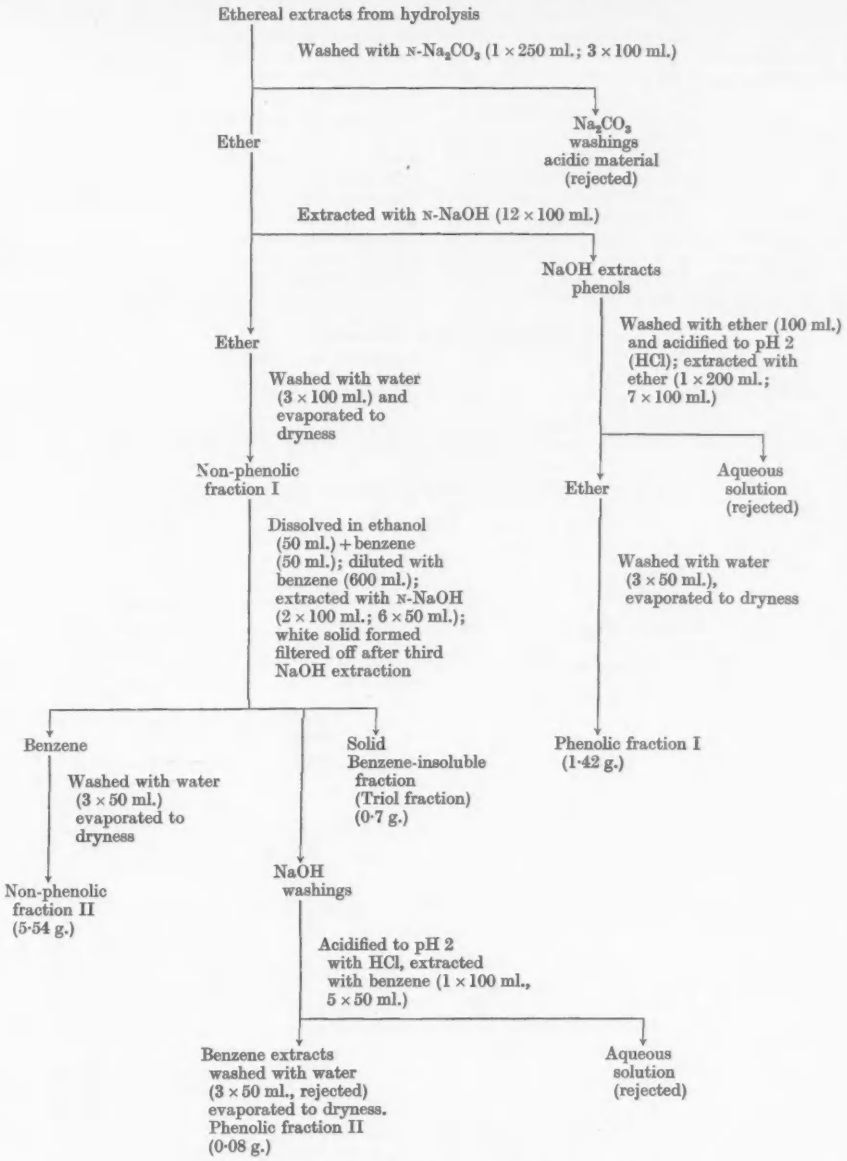

Ethereal extracts from hydrolysis
Washed with N-Na_2CO_3 (1 × 250 ml.; 3 × 100 ml.)
Ether
Na_2CO_3 washings acidic material (rejected)
Extracted with N-NaOH (12 × 100 ml.)
NaOH extracts phenols
Ether
Washed with water (3 × 100 ml.) and evaporated to dryness
Washed with ether (100 ml.) and acidified to pH 2 (HCl); extracted with ether (1 × 200 ml.; 7 × 100 ml.)
Non-phenolic fraction I
Ether
Aqueous solution (rejected)
Dissolved in ethanol (50 ml.) + benzene (50 ml.); diluted with benzene (600 ml.); extracted with N-NaOH (2 × 100 ml.; 6 × 50 ml.); white solid formed filtered off after third NaOH extraction
Washed with water (3 × 50 ml.), evaporated to dryness
Benzene
Solid Benzene-insoluble fraction (Triol fraction) (0·7 g.)
Phenolic fraction I (1·42 g.)
Washed with water (3 × 50 ml.) evaporated to dryness
NaOH washings
Non-phenolic fraction II (5·54 g.)
Acidified to pH 2 with HCl, extracted with benzene (1 × 100 ml., 5 × 50 ml.)
Benzene extracts washed with water (3 × 50 ml., rejected) evaporated to dryness. Phenolic fraction II (0·08 g.)
Aqueous solution (rejected)

Fig. 2. Separation of phenolic and non-phenolic fractions.

Uranediol diacetate. The mother liquors of fraction *BB*1 after further chromatography and crystallization from hexane and from aqueous methanol yielded some uranediol diacetate. Fraction *BA* and all the material from fraction *BB* which had not been purified as uranediol diacetate or compound *MU* were combined and hydrolysed with KOH; the alcohols were submitted to the Marker digitonin separation (as described on p. 701). Uranediol was obtained pure in this way very much more easily than by chromatography and crystallization of the acetates.

Compound MW diacetate. Fraction *BC* (169 mg.) was recrystallized twice from aqueous methanol, once from aqueous ethanol and twice from hexane to give a product, rectangular leaflets, m.p. 175·5–178°, provisionally called *compound MW diacetate.* (Found after subliming at 150–180°/1 μ., C, 74·8; H, 9·7. $C_{25}H_{38}O_4$ requires C, 74·6; H, 9·5. $C_{25}H_{40}O_4$ requires C, 74·2; H, 10·0%. $[\alpha]_D^{19°}$ −54·0±1·1° in $CHCl_3$ (*c*, 1·03).) Insufficient pure material was available for a duplicate determination of the rotation, but since the first determination gave an unexpectedly large negative value, a second determination was carried out, using less pure material of m.p. 173–176°. The value found, $[\alpha]_D^{19°}$ −46·0 ±0·6° in $CHCl_3$ (*c*, 1·00), was in rough agreement with the previous value. *MW* diacetate gave a barely perceptible colour with tetranitromethane in $CHCl_3$.

chromatographed on Al_2O_3. The material eluted with light petroleum-benzene mixtures (70:30–50:50), after crystallization from $CHCl_3$-methanol, formed leaflets, m.p. 214–218° (softening from 209°). This product gave a considerable depression in melting point on admixture with uranediol dibenzoate of m.p. 211–213°. The product has $[\alpha]_D^{20°}$ −24·9 ±0·5° in $CHCl_3$ (*c*, 0·82). The ultraviolet absorption showed maxima at 228 and 272 mμ.; $E^{1\%}_{1\,cm.}$ 317 and 20·2. These values are far too low for the dibenzoate of a C_{21} diol; Pearlman (1944) found $E^{1\%}_{1\,cm.}$ 525 and 37·6 for the corresponding maxima for 5α-pregnane-3β:20β-diol dibenzoate. Cholestan-3β-ol benzoate (mol.wt. 492) had $\lambda_{max.}$ 228 and 272 mμ., $E^{1\%}_{1\,cm.}$ 284 and 17·1 (unpublished observations by Mr H. S. Wiggins). The $E^{1\%}_{1\,cm.}$ values calculated from this for a C_{21} acetate-benzoate of mol.wt. 466 are 300 and 18·1, with

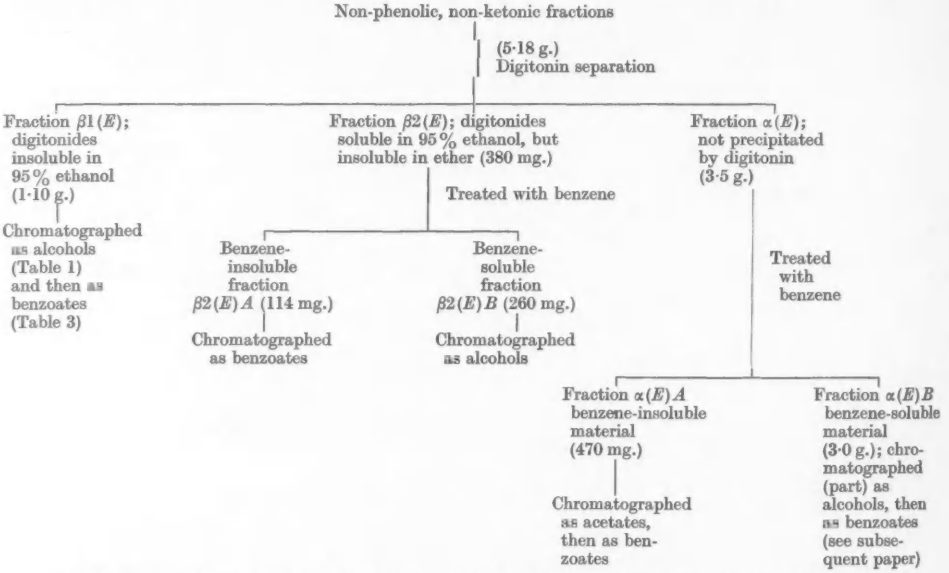

Non-phenolic, non-ketonic fractions
(5·18 g.)
Digitonin separation
Fraction β1(E); digitonides insoluble in 95% ethanol (1·10 g.)
Fraction β2(E); digitonides soluble in 95% ethanol, but insoluble in ether (380 mg.)
Fraction α(E); not precipitated by digitonin (3·5 g.)
Chromatographed as alcohols (Table 1) and then as benzoates (Table 3)
Treated with benzene
Benzene-insoluble fraction β2(E)A (114 mg.)
Benzene-soluble fraction β2(E)B (260 mg.)
Chromatographed as benzoates
Chromatographed as alcohols
Treated with benzene
Fraction α(E)A benzene-insoluble material (470 mg.)
Fraction α(E)B benzene-soluble material (3·0 g.); chromatographed (part) as alcohols, then as benzoates (see subsequent paper)
Chromatographed as acetates, then as benzoates

Fig. 3. Fractionation of non-phenolic non-ketonic fraction following Marker, Rohrmann & Wittle (1938). Outline of method.

Hydrolysis of compound MW diacetate with potassium carbonate. The diacetate (8 mg.) in methanol (0·5 ml.) was hydrolysed by refluxing with K_2CO_3 (10 mg.) in water (0·1 ml.) for 1 hr. The usual working-up yielded a product which was readily soluble in ethanol, sparingly soluble in hexane, more soluble in ether; this on crystallization from hexane-ether gave rosettes of very small needles, m.p. 152–156°. This material gave no precipitate with digitonin in 90% ethanol, and no colour with tetranitromethane in $CHCl_3$. It dissolved in conc. H_2SO_4 to give, after a few seconds, an orange-red colour with a slight green fluorescence; in the Liebermann-Burchard reaction both layers developed a golden-yellow colour in a few seconds. Evidence presented later indicates that this material may have been compound *MW* monoacetate.

The mother liquors of the crystallization from which *MW* diacetate had been obtained were hydrolysed with K_2CO_3 in aqueous methanol; the crude product was benzoylated and which the experimental values for the benzoylation product of m.p. 214–218° are in fair agreement.

Hydrolysis of MW esters with potassium hydroxide. The presumed acetate-benzoate of m.p. 214–218° (18 mg.) was refluxed for 2 hr. with excess KOH in aqueous methanol. The usual working-up yielded 10 mg. solid which, after crystallization from ether-light petroleum, gave small needles m.p. 215° (after subliming in needles from 190°). Mixed m.p. with uranediol, 212–215°. The product gave, during heating on the Kofler block, the characteristic smell of heated uranediol. Concentration of the mother liquors gave a further quantity of the same material. $[\alpha]_D^{20°}$ +2·9 ±1·0° in $CHCl_3$ (*c*, 0·62). Two previous determinations of the rotation of uranediol gave $[\alpha]_D$ +2·1±0·8° and +3·7±0·8° in $CHCl_3$.

The hydrolysis product on acetylation gave an acetate which on crystallization from methanol formed prisms, m.p. 163–165°; not depressed on admixture with authentic uranediol diacetate.

The presumed monoacetate of compound *MW* (m.p. 152–156°) was similarly hydrolysed with KOH in aqueous methanol. The product had m.p. 214–217°, not depressed by admixture with uranediol.

Separation of non-phenolic non-ketones by digitonin in ethanol and ether

The non-ketonic material from batch *B* (5·18 g.) was dissolved in boiling ethanol (95% v/v, 450 ml.) and a hot solution of digitonin (15 g.) in ethanol (95% v/v, 900 ml.) was added (cf. Marker, Rohrmann & Wittle, 1938). The separations are outlined in Fig. 3. The mixture was cooled to 15–20° and after 3 hr. the precipitate which formed was filtered off, washed with ethanol (95% v/v) and decomposed with pyridine and ether. The digitonin-free product (fraction $\beta 1(E)$) weighed 1·10 g. The ethanolic filtrate was concentrated *in vacuo* on the water bath until solid began to separate (volume of solution, approx. 40 ml.). Dry ether (800 ml.) was added and the precipitate which formed was filtered off after standing 1 hr., and washed with ether. This second digitonide precipitate was decomposed like the first, giving fraction $\beta 2(E)$ (380 mg.). The ethereal filtrate was evaporated to dryness giving fraction $\alpha(E)$ (3·5 g.).

Chromatography of fraction $\beta 1(E)$ as free alcohols

The fraction (1·10 g.) was dissolved as much as possible in benzene-ether (80:20), filtered from a little undissolved material, and chromatographed on Al_2O_3. The results of this and four subsequent chromatograms on the material as free alcohols are shown in Table 1.

Uranediol. Fraction III*B* (Table 1) after crystallization from aqueous ethanol or from ethyl acetate-light petroleum gave uranediol, 50 mg., m.p. 216–219°, not depressed by admixture with an authentic sample obtained from the sulphate (Klyne, 1948). Further quantities (30 mg.) were obtained from fractions II*C* and *D* by chromatography and crystallization.

5α-Pregnane-3β:20β-diol. Each of the seven groups of fractions IV*B–H* (Table 1) on crystallization from ethyl acetate-light petroleum yielded a product of melting point between 186 and 194°, total wt. 170 mg. The three best fractions were combined and crystallized again from the

Table 1. *Chromatography of fraction $\beta 1(E)$ as alcohols*

Run	Eluent	Eluate: Fraction no.	Eluate: Weight (mg.)	Eluate: Appearance	Melting point
	Chromatogram I: Fraction $\beta 1(E)$; 30 g. Al_2O_3; each run 100 ml. of eluent				
1–3	Benzene-ether (80:20)	I*A*	352	Gum, see chromatogram II	—
4	Benzene-ether (80:20)	I*B*	219	Solid, see chromatogram III	182–192°
5–9	Benzene-ether (80:20)	I*C*	376	Solid, see chromatogram IV	155–175°
10–12	Benzene-ether (50:50)	I*D*	53	Gum } see chromatogram V	—
13–14	Ether	I*E*	26	Gum }	
15–17	Ether-methanol (50:50) and methanol	I*F*	240	Gum	—
	Chromatogram II: Fraction I*A*; 10·5 g. Al_2O_3; each run 35 ml. of eluent				
1–5	Benzene-ether (90:10)	II*A*	133	Gum	—
6–7	Benzene-ether (90:10)	II*B*	25	Crystals	170–190°
8–9	Benzene-ether (80:20)	II*C*	32	Crystals	203–210°
10	Benzene-ether (80:20)	II*D*	8	Crystals	193–205°
11–25	Benzene-ether (60:40 and 20:80) and ether	II*E*	12	Gum	—
	Chromatogram III: Fraction I*B*; 18 g. Al_2O_3; each run 20 ml. of eluent				
1–11	Benzene-ether (up to 25% ether)	III*A*	21	Gum	—
12–13	Benzene-ether (70:30)	III*B*	80	Crystals	207–217°
14	Benzene-ether (70:30)	III*C*	29	Crystals	190–200°
15	Benzene-ether (70:30)	III*D*	33	Crystals	145–160°
16–18	Benzene-ether (70:30)	III*E*	39	Crystals	155–180°
19–20	Benzene-ether (60:40)	III*F*	20	Gum	—
	Chromatogram IV: Fractions I*C* + III*E*; 27 g. Al_2O_3; each run 40 ml. of eluent				
1–8	Benzene-ether (85:15 and 80:20)	—	Nil		—
9–11	Benzene-ether (75:25)	IV*A*	113	Crystals	150–180°
12–15	Benzene-ether (75:25) }	IV*B–H*	309	Seven groups of crystalline fractions	165–190° mainly 180–190°
16–23	Benzene-ether (70:30) }				
24–25	Benzene-ether (60:40)	IV*J*	19	Crystals	150–165°
	Chromatogram V: Fractions I*D* + I*E* + II*E*; 2·2 g. Al_2O_3; each run 7 ml. of eluent				
1–2	Benzene-ether (66:33)	V*A*	23	Gum	—
3–5	Benzene-ether (66:33)	V*B*	13	Gum	—
6–9	Benzene-ether (33:66)	V*C*	5	Solid	90–110°
10–12	Ether				
13–26	Ethanol-methanol mixtures and methanol }	V*D*	12	Gum	—

Table 2. *Identification of urinary 5α-pregnane-3β:20β-diol*

Diol	Urinary compound		Authentic 5α-pregnane-3β:20β-diol*
M.p.	194·5–196°		194–195°
Mixed m.p.		195–198°	
$[\alpha]_D$ in $CHCl_3$	−0·7±0·7° (*c*, 1·11)		+2·9±0·9° (*c*, 1·02)
Analysis	C, 78·7; H, 11·2%		C, 78·7; H, 11·3% (calc. for $C_{21}H_{36}O_2$)
Diacetate			
M.p.	140·5–141·5°		141–142°
Mixed m.p.		139·5–141°	
$[\alpha]_D$ in $CHCl_3$	+20·8±0·8° (*c*, 1·00)		+22° (*c*, 5·2)
Analysis	C, 74·0; H, 10·1%		C, 74·2; H, 10·0% (calc. for $C_{25}H_{40}O_4$)
Dibenzoate			
M.p.	236·5–237·5°		237·5–239°
Mixed m.p.		235·5–237·5°	
$[\alpha]_D$ in $CHCl_3$	−7·7±0·4° (*c*, 2·04)		−10·1±0·5° (*c*, 2·08)
Analysis	C, 79·6; H, 8·6%		C, 79·5; H, 8·4% (calc. for $C_{35}H_{44}O_4$)

* Data for the diol are from Klyne & Miller (1950); data for the diacetate from Klyne & Barton (1949); data for the *dibenzoate* are new.

Table 3. *Chromatography of parts of fraction β1 (E) as benzoates*

Run	Eluent	Fraction no.	Eluate: Weight (mg.)	Eluate: Appearance*	Melting point
	Chromatogram VI: Benzoylated mother liquors of 5α-pregnane-3β:20β-diol from fractions IV*B–H* (Table 1). 12 g. Al_2O_3; each run 20 ml. of eluent				
1–8	Light petroleum-benzene (90:10)	VI*A*	82	Crystals (5α-pregnane-3β:20β-diol)	230–240°
9–13	Light petroleum-benzene (85:15)	VI*B*	32	Solid (5α-pregnane-3β:20α-diol)	158–170°
14–16	Light petroleum-benzene (80:20)				
17–19	Light petroleum-benzene (70:30)	VI*C*	4	Trace gum	—
20–25	Light petroleum-benzene (50:50)	VI*D*	35	Solid (compound *MM*)	145–157°
26–29	Light petroleum-benzene (25:75)	VI*E*	23	Gum	—
30–32	Benzene				
	Chromatogram VII: Fractions II*D*, III*C*, *D*, *F*, IV*A*, Table 1 (intermediate between uranediol and 5α-pregnane-3β:20β-diol in alcohol chromatograms); benzoylated; 15 g. Al_2O_3; each run 25 ml. of eluent				
1–3	Light petroleum-benzene (95:5)	VII*A*	10	Gum	—
4–13	Light petroleum-benzene (90:10)	VII*B*	111	Solid (5α-pregnane-3β:20β-diol)	234–238°
14–16	Light petroleum-benzene (85:15)	VII*C*	13	Solid (as VII*B*)	223–232°
17	Light petroleum-benzene (85:15)	VII*D*	18	Solid	120–216°
18–20	Light petroleum-benzene (80:20)				
21	Light petroleum-benzene (70:30)				
22–25	Light petroleum-benzene (70:30)	VII*E*	54	Solid (uranediol)	202–211°
26	Light petroleum-benzene (70:30)	VII*F*	16	Solid	135–197°
27	Light petroleum-benzene (60:40)				
28–32	Light petroleum-benzene (60:40)	VII*G*	29	Solid (compound *MM*)	183–196°
33–40	Light petroleum-benzene (50:50 and 25:75) and benzene	VII*H*	22	Gum	—

* Compounds present, as benzoates, shown in parentheses.

same solvent mixture yielding 40 mg. 5α-pregnane-3β:20β-diol. The properties of this product and its diacetate, and of authentic 5α-pregnane-3β:20β-diol are listed in Table 2.

Chromatography of fraction β1 (E) as acetates

Fraction I*F* (Table 1) was the material eluted last from Al_2O_3: it was expected that this would consist of triols. It was acetylated and chromatographed on Al_2O_3. Light petroleum-benzene mixtures (60:40 and 50:50) eluted solid fractions (56 mg.) with melting points in the range of 126–155°. This material was combined and recrystallized twice from methanol (0·5 and 0·3 ml.) and once from light petroleum (0·5 ml.) to yield 5α-pregnane-3β:16α:20β-triol triacetate, m.p. 164–168° (not depressed by admixture with authentic material), $[\alpha]_D^{20°}$ −51·3±0·6° in $CHCl_3$ (*c*, 1·35).

m.p. 163–164°, which depressed the m.p. of 5α-pregnane-3β:20α-diol dibenzoate. A second recrystallization from the same mixture (about 4 ml.) gave rectangular leaflets (15 mg.) which were a polymorphic modification of the compound, m.p. 191·5–192·5°. (Found: C, 79·7; H, 8·1. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4. $C_{35}H_{42}O_4$ requires C, 79·8; H, 8·0. $C_{33}H_{40}O_4$ requires C, 79·2; H, 8·1. $C_{33}H_{38}O_4$ requires C, 79·5; H, 7·7%.) The ultraviolet absorption of *MM* benzoate was compatible with its formulation as the dibenzoate of a C_{21} diol; $\lambda\lambda_{max.}$ 230 and 273 mμ., $E^{1\%}_{1\,cm.}$ 527 and 39·0. Pearlman (1944) found for 5α-pregnane-3β:20β-diol dibenzoate $\lambda\lambda_{max.}$ 231 and 272 mμ., $E^{1\%}_{1\,cm.}$ 525 and 37·6.

Recrystallization of fraction VII*G* from $CHCl_3$-methanol mixture yielded a further 18 mg. of compound *MM* benzoate.

Table 4. *Identification of urinary 5α-pregnane-3β:20α-diol*

	Urinary compound		Authentic 5α-pregnane-3β:20α-diol*
Dibenzoate			
M.p.	170·5–172°		170–171°
Mixed m.p.		168·5–169·5°	
$[\alpha]_D^{20°}$ in $CHCl_3$	+27·7±0·7° (*c*, 0·84)		+27·8±0·2° (*c*, 2·10)
Diol			
M.p.	217–218°		218–220°
Mixed m.p.		217–218·5°	
$[\alpha]_D$ in $CHCl_3$	+20·0±1·4° (*c*, 0·64)		+22·6±1·3° (*c*, 0·93)

* 5α-*Pregnane*-3β:20α-*diol dibenzoate* (authentic). (Found: C, 79·2; H, 8·3. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4%.)

Chromatography of fraction β1 (E) as benzoates

The mother liquors (116 mg.) from the crystallization of 5α-pregnane-3β:20β-diol in fractions IV *B–H* (Table 1) were benzoylated and the product (190 mg.) chromatographed on Al_2O_3. (Chromatogram VI, Table 3.)

The mother liquors from the crystallization of uranediol were combined with fractions II*D*, III*C*, *D*, *F*, and IV*A* (Table 1) and benzoylated. The product (260 mg.) was chromatographed on Al_2O_3. (Chromatogram VII, Table 3.)

From these chromatograms four benzoates were readily separated, namely those of 5α-pregnane-3β:20α- and -3β:20β-diols, uranediol and a new compound *MM*.

5α-*Pregnane*-3β:20β-*diol*. Fraction VI*A* on recrystallization twice from $CHCl_3$-methanol (25:75) yielded needles, m.p. 236·5–237·5°. Details of the comparison with authentic 5α-pregnane-3β:20β-diol dibenzoate and analysis are given in Table 2. Further quantities were obtained from fractions VII*B*, *C* and VIII *B*, *C* by crystallization.

5α-*Pregnane*-3β:20α-*diol*. Fraction VI*B* was recrystallized twice from $CHCl_3$-methanol (20:80) yielding 12 mg. of prisms which proved to be the dibenzoate of 5α-pregnane-3β:20α-diol. The properties of this product, of its parent diol and of authentic material are listed on Table 4. Dr K. Dobriner (Sloan-Kettering Institute, New York) kindly determined the infrared absorption of the dibenzoate obtained from the urinary material and stated that it was identical with that of the authentic material 'with the exception of a small band at 1040 cm.$^{-1}$, which may be due to some impurity'.

Compound MM. Recrystallization of fraction VI*D* (Table 3) from $CHCl_3$-methanol mixture (20:80, about 6 ml.) yielded 24 mg. of *compound MM dibenzoate*, plates,

Hydrolysis of the benzoate (24 mg.) by refluxing with methanolic KOH yielded 13·5 mg. of a product which was recrystallized twice from ethyl acetate-light petroleum to yield compound *MM* (5·3 mg.), rosettes of elongated prisms, m.p. 184–185° after subliming in needles from 162°. Compound *MM* gave a precipitate with digitonin in 95% ethanol, but gave no colour with tetranitromethane. In the Liebermann-Burchard test it rapidly gave a reddish brown colour in the conc. H_2SO_4 layer; the $CHCl_3$ layer remained colourless.

Compound *MM* was acetylated and the product was chromatographed on Al_2O_3 and recrystallized from light petroleum giving needles, m.p. 108–109·5°.

Table 5 shows a comparison of the properties of compound *MM* and its derivatives with those of pregnane-3β:20β-diol and 5α-pregn-16-ene-3β:20β-diol. *MM* is clearly not identical with pregnane-3β:20β-diol. Dr K. Dobriner kindly examined the infrared absorption of *MM* acetate; he stated that it was not identical with that of 5α-pregn-16-ene-3β:20β-diol diacetate, but that compound *MM* may contain an olefinic double bond.

Uranediol. Fraction VII*E* (Table 3) was recrystallized twice from $CHCl_3$-methanol (20:80) to yield leaflets (30 mg.), m.p. 209–210°, not depressed by admixture with an authentic sample of uranediol dibenzoate, $[\alpha]_D^{20°}$ +18·6°±0·6° (in $CHCl_3$, *c*, 1·05). The authentic sample of the *dibenzoate* had m.p. 211–213°. $[\alpha]_D$ +22·4°±1·2°. (Found: C, 79·4; H, 8·1. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4%.) Ultraviolet absorption, $\lambda_{max.}$ 230 and 272 mμ., log $\epsilon_{max.}$ 4·43 and 3·26.

Other compounds. Fraction II*A* (Table 1; 133 mg.) consisted of material more easily eluted from Al_2O_3 than the pregnanediol isomers. It was chromatographed again on Al_2O_3 but, since no apparent separation occurred, the bulk

of the material was combined and benzoylated. Chromatography of this product on Al_2O_3 yielded chiefly gums, but small quantities of two solid compounds were obtained. The first solid fraction (4 mg.) eluted with light petroleum-benzene (90:10) was recrystallized twice from methanol to yield plates of m.p. 167–171° (compound *MR* benzoate). The second compound was eluted with light petroleum-benzene (60:40) mixtures and had m.p. 185–188° after being recrystallized twice from methanol (compound *MS* benzoate). Neither compound was identical with any known benzoate.

eluted *BR* (50 mg.) and methanol *BS* (50 mg.). Since uranediol and 5α-pregnane-3β:20β-diol were subsequently obtained more easily by chromatography of their benzoates, it seems unnecessary to give details of their isolation as diols from these fractions.

The mother liquors from the crystallization of 5α-pregnane-3β:20β-diol and uranediol were combined with similar material from the urine of batch *D* and benzoylated. The product (179 mg.) was chromatographed on Al_2O_3. Light petroleum-benzene mixtures (85:15) eluted 70 mg. of solid which was recrystallized from $CHCl_3$-methanol to yield 5α-

Table 5. *Comparison of compound MM with pregnane-3β:20β-diol and 5α-pregn-16-ene-3β:20β-diol*

	Compound *MM*		Pregnane-3β:20β-diol	5α-Pregn-16-ene-3β:20β-diol†
Dibenzoate				
M.p.	163–164° and 191·5–192·5°		Gum	—
$[\alpha]_D$ in $CHCl_3$	−14·6±0·4° (*c*, 1·04)		−13° (calc.)*	—
Analysis	C, 79·7; H, 8·1%		C, 79·5; H, 8·4% (calc. for $C_{35}H_{44}O_4$)	C, 79·8; H, 8·0% (calc. for $C_{35}H_{42}O_4$)
Diol				
M.p.	184–185°		177–178°‡	188–190°
Mixed m.p.		169–178°		
$[\alpha]_D$ in EtOH	−5·9±0·7° (*c*, 0·55)		+4·9±0·4° (*c*, 1·91)	—
Diacetate				
M.p.	108–109·5°		108–110°‡	102–104°
Mixed m.p.		104–110°		
$[\alpha]_D$ in $CHCl_3$	−14·0±0·7° (*c*, 0·59)		+32·9±0·8° (*c*, 1·04)	—

* From tables of standard values given by Barton & Klyne (1948).

† Data from Marker, Turner, Wagner, Ulshafer, Crooks & Wittle (1941).

‡ Authentic pregnane-3β:20β-diol was prepared from pregnan-3β-ol-20-one by reduction with $LiAlH_4$ as described for pregn-5-en-3β-ol-20-one by Klyne & Miller (1950) except that the reaction mixture was not heated. The 3β:20α- and 3β:20β-diol dibenzoates were separated by chromatography. Marker, Kamm & Jones (1937) and Marker, Kamm, Wittle *et al.* (1937) give for pregnane-3β:20β-diol m.p. 174°, and for its diacetate m.p. 111°.

The bulk of the material from chromatogram V (fractions V*A*, *B* and *C*) was benzoylated and chromatographed on Al_2O_3 (chromatogram VIII). Nearly all the material was eluted with light petroleum-benzene mixtures. The first four 10% benzene runs eluted a little 5α-pregnane-3β:20β-diol dibenzoate (fractions VIII*B* and *C*). Runs 6 and 7 (10% benzene) eluted 11 mg. crystalline material which after two recrystallizations from $CHCl_3$-methanol formed plates m.p. 185·5–187°. This material was apparently different from all the other benzoates isolated in this work and has been called provisionally compound *MN* benzoate. It gave a depression in melting point on admixture with the benzoate of similar melting point (*MS*) from fraction II*A* above. Lack of material has prevented further study. Later runs with increasing benzene content (10–50%) eluted material melting chiefly below 100°, from which no homogeneous product has been obtained.

Fraction β2(E)

This fraction (380 mg.) was treated with benzene (50 ml.) and separated into a benzene-insoluble fraction *β2(E) A* (114 mg.) and a benzene-soluble fraction *β2(E) B* (266 mg.).

Benzene-soluble fraction. Fraction *β2(E) B* was chromatographed on Al_2O_3: benzene-ether mixtures (75:25) eluted first fraction *BP* (46 mg., fairly pure uranediol of m.p. 205–207°) and then *BQ* (70 mg.); this was a mixture containing uranediol and 5α-pregnane-3β:20β-diol; acetone eluted pregnane-3β:20β-diol dibenzoate. Further solid fractions were eluted with 20% benzene (10 mg.) and 30% benzene (34 mg.); these on recrystallization yielded the dibenzoates of 5α-pregnane-3β:20α-diol and uranediol, respectively.

Fraction *BR* was benzoylated and the product chromatographed on Al_2O_3. The first solid fraction eluted (3 mg.) with light petroleum-benzene (80:20) was recrystallized from $CHCl_3$-methanol to yield plates, m.p. 181–184°. (Mixed melting point with compound *MN* benzoate—from chromatogram VIII—183–187°.) Fraction *BS* was benzoylated and the product chromatographed on Al_2O_3. Light petroleum-benzene mixtures (80:20 and 70:30) eluted solid fractions (79 mg.) with indefinite melting points in the range 100–140°. Crystallization of these fractions has not, so far, yielded a homogeneous product.

Benzene-insoluble fraction. Fraction *β2(E) A* was benzoylated and the product (183 mg.) chromatographed on Al_2O_3 (chromatogram IX). The principal solid fractions, eluted with light petroleum-benzene mixtures, were as follows. Fraction IX*B*, eluted with 20% benzene (8 mg.) contained 5α-pregnane-3β:20β-diol dibenzoate. Fraction IX*D* (25 and 30% benzene; 32 mg.) and fraction IX*F* (40% benzene, 54 mg.) yielded the tribenzoate of 5α-pregnane-3β:16α:20β-triol after one crystallization from methanol as elongated prisms m.p. 211–214°, not depressed by admixture with an authentic sample. The intermediate fraction IX*E* (30 and 40% benzene, 48 mg.) consisted of less pure 5α-pregnane-3β:16α:20β-triol tribenzoate.

Fraction α(E)

This was separated, by warming with 30 ml. benzene and filtering, into two fractions, *α(E)B* (soluble in benzene, 3·0 g.) and *α(E)A* (insoluble in benzene, 470 mg.). Fraction *α(E)B* has been kept for further study; fraction *α(E)A* was treated like the triol fraction from batch *D* (see below).

The triol fraction

Separation of acetates by crystallization from methanol. The triol fraction from batch *D* (0·7 g.) (see p. 698) was acetylated and the product submitted to the Girard separation. The ketonic fraction (28 mg.) has not been further investigated. The non-ketonic fraction (767 mg.) was chromatographed on Al_2O_3 (activity II). Hexane-benzene mixtures (60:40 and 40:60) eluted white, crystalline fractions, which were all apparently mixtures. These fractions (*TA*) were combined (440 mg.) and recrystallized twice from methanol (2 ml. and 1 ml.) (cf. Marker, Kamm, Crooks *et al.* 1938) giving 177 mg. of needles, m.p. 158–160°. After a further recrystallization from methanol this material had m.p. 164–166°. The mixed m.p. with a sample of 5α-pregnane-3β:16α:20β-triol triacetate of Haslewood *et al.* (1934), m.p. 163–165°, kindly supplied by Prof. Marrian, was 163–164°. The methanol mother liquors from the first crystallization of the fractions *TA* (1·5 ml.) were treated with water (0·5 ml.), warmed to dissolve the precipitate which formed and then cooled. The crystalline solid which separated (200 mg., m.p. 134–137°) was recrystallized from 70% methanol-30% water giving uranetriol triacetate, small plates, m.p. 135–136°. Marker, Kamm, Crooks *et al.* (1938) give m.p. 136°. This substance sublimed at 180°/0·5 μ. (Found: C, 69·6; H, 9·2. Calc. for $C_{27}H_{42}O_6$: C, 70·1; H, 9·2%.) $[\alpha]_D^{21°}$ +7·8°±1·3° in $CHCl_3$ (*c*, 1·00); $[\alpha]_D^{21°}$ +0·0°±0·9° in ethanol (*c*, 1·01). $[M]_D$ in $CHCl_3$, +37.

Separation of uranetriol triacetate by crystallization from light petroleum. It was expected that fraction *α(E)A* from batch *B* (see above) would consist of triols; this fraction (470 mg.) was therefore acetylated and the acetates (560 mg.) chromatographed on Al_2O_3. Light petroleum-benzene mixtures and benzene eluted 430 mg. gum (fraction *TB*); benzene-ether and ether eluted approx. 130 mg. yellow gum (fraction *TC*). Fraction *TB* was chromatographed again yielding 370 mg. almost colourless, partly crystalline material (some fractions melted at approx. 80–90°). This material failed to crystallize from methanol when the Marker fractionation of triacetates was attempted. However, when the material was allowed to stand at 0° with 1 ml. light petroleum (b.p. 40–60°) for a week, crystals formed. The supernatant liquid was removed and the crystals triturated twice with 0·5 ml. light petroleum. The final crystalline product (103 mg.; m.p. 123–125°) was crystallized three times from methanol and once from ethyl acetate-light petroleum yielding a compound m.p. 153–154° (15 mg.). (Found, on material sublimed at 170–180°/0·1 μ., C, 69·7; H, 8·8. Calc. for $C_{27}H_{42}O_6$: C, 70·1; H, 9·1%) $[\alpha]_D^{22°}$ +12·2°±0·6° in $CHCl_3$ (*c*, 1·00). Subsequent comparison of the infrared absorption of this material (form *b*) and of our previous uranetriol triacetate (form *a*; m.p. 135–136°) by Dr K. Dobriner and Mrs P. Humphries showed that the two substances were identical. Mixed melting point determinations with uranetriol triacetate (form *a*) were inconclusive. Form *a* melted at about 128–131° and remained liquid on further heating; form *b* melted at 151–153°.

Separation of uranetriol tribenzoate by chromatography. Mother liquors from the crystallization of uranetriol triacetate from urine of batch *B* were hydrolysed, benzoylated and then chromatographed as their benzoates (293 mg.). The first two fractions eluted with light petroleum-benzene mixture (60:40) were gums (53 mg.). These were followed by four solid fractions (108 mg.), m.p. 199–230°, eluted with the same mixture. Repeated recrystallization of these solid fractions from $CHCl_3$-methanol gave *uranetriol tribenzoate* as fine needles, m.p. 233·5–235°, undepressed by a specimen prepared from an authentic sample of uranetriol. $[\alpha]_D^{20°}$ + 82·5°±0·4° in $CHCl_3$. Another sample had $[\alpha]_D$ +80·8±0·4° in $CHCl_3$ (*c*, 1·02) $[M]_D$ +524. (Found, C, 77·3; H, 7·5. $C_{42}H_{46}O_6$ requires C, 77·7; H, 7·5%.) Ultraviolet absorption, $\lambda_{max.}$ 231 and 272 mμ., log $\epsilon_{max.}$ 4·61 and 3·48.

Uranetriol. Samples of the two forms of the triacetate (*a*, of m.p. 135–136°; *b*, of m.p. 153–154°) were hydrolysed with boiling aqueous methanolic KOH. The products obtained by the usual working-up and crystallized from acetone-methanol were solids of no definite crystalline appearance. Triol from the triacetate (form *a*) had m.p. 296–298° (uncorr.) after subliming to long needles at 230° on the Kofler block. $[\alpha]_D^{20°}$ +29·8±3·1° in ethanol (*c*, 0·41). The triol from the triacetate (form *b*) had m.p. 297–299° (uncorr.) after subliming at 235°, $[\alpha]_D^{22°}$ +28·5±1·5° in ethanol (*c*, 0·53); another sample $[\alpha]_D^{20°}$ +26·2±1·4° (*c*, 0·56); $[M]_D$ +94. Mixed melting point of triols from the two forms of the triacetate, 294–297° (uncorr.). Marker, Kamm, Crooks *et al.* (1938) give m.p. 295–300° for uranetriol. Uranetriol gave no precipitate with digitonin in 90% (v/v) ethanol and no coloration with tetranitromethane. In conc. H_2SO_4 it gave an orange solution with a yellow-green fluorescence and in the Liebermann-Burchard test an orange colour.

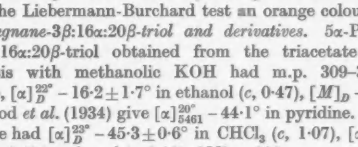
5α-Pregnane-3β:16α:20β-triol and derivatives. 5α-Pregnane-3β:16α:20β-triol obtained from the triacetate by hydrolysis with methanolic KOH had m.p. 309–310° (uncorr.), $[\alpha]_D^{22°}$ −16·2±1·7° in ethanol (*c*, 0·47), $[M]_D$ −54. Haslewood *et al.* (1934) give $[\alpha]_{5461}^{20°}$ −44·1° in pyridine. The triacetate had $[\alpha]_D^{23°}$ −45·3±0·6° in $CHCl_3$ (*c*, 1·07), $[\alpha]_D^{25°}$ −45·7°±0·6° in ethanol (*c*, 1·01); $[M]_D$ −208.

The tribenzoate formed long thin needles from $CHCl_3$-methanol, m.p. 211–213° (Marker, Kamm, Wittle, Oakwood & Lawson, 1938, give m.p. 218°) $[\alpha]_D^{20°}$ −14·1±0·8° in $CHCl_3$ (*c*, 1·06), $[M]_D$ −91. (Found: C, 77·3; H, 7·5%. Calc. for $C_{42}H_{46}O_6$: C, 77·7; H, 7·5%.)

DISCUSSION

Configurations at $C_{(20)}$

Configurations at $C_{(20)}$ in the steroid skeleton in this paper are allotted following Fieser & Fieser (1948, 1949) who first related the stereochemistry of $C_{(20)}$ to that of the nucleus. They proposed a convention for representing side-chain configurations in which $C_{(21)}$ is imagined as lying in the rearmost possible position, as in Fischer projections of aliphatic compounds:



20α-Hydroxysteroid 20β-Hydroxysteroid

They produced evidence which indicated that, for compounds carrying a 17α-hydroxyl group, the 20α- and 20β-configurations were as in III and IV. This allotment of configurations was extended to compounds not carrying a 17α-hydroxyl group by Sarett (1949) and by Klyne & Barton (1949). The arguments of Fieser & Fieser regarding these configurations were recently called in question by Lardon & Reichstein (1951); evidence confirming the Fieser configurations was subsequently discussed by Klyne (1951).

The occurrence of 20β-hydroxysteroids in urine

The chief result of this work to date is the confirmation of the finding by Bauld & Heard (1940) that 5α-pregnane-3β:20β-diol is one of the major steroids of mares' pregnancy urine. No 20β-hydroxysteroid has ever been reported in human pregnancy urine, in spite of the fact that the 'pregnanediol' fractions have been studied by many workers. (For review, see Pearlman, 1948.) Mares' pregnancy urine contains another 20β-hydroxysteroid—the 5α-pregnane-3β:16α:20β-triol isolated by Haslewood *et al.* (1934), the structure of which was finally elucidated by Hirschmann *et al.* (1949). Two triols of somewhat similar structure have recently been identified in the urine of a boy with an adrenocortical tumour (Hirschmann & Hirschmann, 1950*a*, *b*). These compounds, pregn-5-ene-3β:16α:20α-triol and -3β:17α:20α-triol, like all other human urinary steroids carrying a hydroxyl group at $C_{(20)}$, have the 20α-configuration.

Marker & Rohrmann (1938) claimed to have isolated 5α-pregnane-3β:20α-diol from pregnant mares' urine, but they obtained this compound only from a fraction which had been epimerized by heating with sodium in xylene. We found a much smaller quantity of 5α-pregnane-3β:20α-diol than of the 20β-isomer, and it is surprising that Marker & Rohrmann did not isolate the 20β-isomer.

At an early stage in this work we suspected that Marker's 3β:20α-diol might be an artifact formed from the 3β:20β-diol by epimerization at $C_{(20)}$. However, our own experiments in which 5α-pregnane-3β:20β-diol was heated with sodium in xylene confirmed the findings of Marker, Kamm, Wittle, Oakwood, Lawson & Laucius (1937), who stated that no appreciable epimerization at $C_{(20)}$ takes place in these conditions.

The present work appears to be the first occasion on which a pair of 20-epimeric hydroxysteroids has been isolated from the same urine. The only other source from which a pair of 20-epimers has been obtained is the adrenal glands, from which Reichstein (1936) and Steiger & Reichstein (1938) isolated 5α-pregnane-3β:17α:20α-triol and -3β:17α:20β-triol ('substances *O* and *J*').

Two 20β-hydroxysteroids have been isolated from ox bile, namely 5α-pregnane-3β:20β-diol (Pearlman, 1944, 1946) and pregnane-3α:20β-diol (from pregnant cows' bile, Pearlman & Cerceo, 1948).

Value of alternative digitonin separations

The Marker digitonin separation using ethanol and ether (Marker, Rohrmann & Wittle, 1938) offers a considerable advantage over the digitonin separation using methanol in that the two principal 3β-hydroxysteroids of the mares' urine extracts (5α-pregnane-3β:20β-diol and uranediol) are partly separated, one being concentrated in each of the two digitonin precipitates of the Marker method. The separation is, however, far from perfect, and before the benzoates were used in chromatography, the separation of the two major constituents in pure condition was difficult, and the separation of the minor constituents almost impossible.

The fact that some minor constituents (compounds *MU*, *MV* and *MW*) have been isolated only from the urine extracts submitted to the methanol-separation, whilst others (compounds *MM*, *MN*, *MR* and *MS*) have been isolated only from the extracts treated by the Marker method may be due to differences in the solubilities of the acetates and benzoates of the compounds, or to chance differences in the composition of the two batches of pooled urine. It is possible that *MU* or *MW* (obtained as their acetates) may be identical with *MN*, *MR* or *MS* (obtained as their benzoates), but the quantities of material available at present do not enable us to test these hypotheses.

Although neither of the two triols (5α-pregnane-3β:16α:20β-triol and uranetriol) is precipitated from pure solution by digitonin in the usual conditions, the fractionation of batch *B* by the Marker digitonin method shows that the 5α-pregnanetriol may be partially precipitated from mixtures by digitonin.

SUMMARY

1. The fractionation of the steroids of pregnant mares' urine into phenols and non-phenols, ketones and non-ketones, and the further separation of some fractions with digitonin are described.

2. The non-phenolic, non-ketonic, digitonin-precipitable fraction has been studied in detail. The isolation of uranediol, 5α-pregnane-3β:20β-diol and 5α-pregnane-3β:20α-diol from this fraction is described. This is the first reported isolation of a pair of 20-epimeric hydroxysteroids from any urine.

3. The isolation of seven apparently new compounds of undetermined structure from the same fraction is described. Compounds *MM*, *MU* and *MW* are probably C_{21} dihydroxysteroids; they were

isolated as their acetates or benzoates. Compound *MW* may be a 17*α*-epimer of uranediol. Compound *MV* is apparently a monohydroxy compound. The three remaining compounds, *MN*, *MR* and *MS*, have been obtained only in traces.

4. The significance of the isolation of 20*β*-hydroxysteroids from mares' urine is discussed. All 20-hydroxysteroids obtained from human urine are 20*α*-compounds.

5. Uranetriol has been obtained from the trihydroxysteroid fraction and studied.

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Studies in Vitamin A

18. THE EFFECT OF VITAMIN A DEFICIENCY ON THE PYRUVATE AND α-KETOGLUTARATE LEVELS OF RAT BLOOD

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The problem of the mode of action of vitamin A in the general metabolic processes of mammals (i.e. other than in specialized functions such as night vision) has not yet been solved. From this point of view, the work of von Euler & Högberg (1939, 1940) is important, for it suggests that vitamin A may function in phosphorylation processes. They found that the blood pyruvate levels of vitamin A-deficient rats were more than three times greater than those of rats maintained on the same diet supplemented with vitamin A (43 μg./ml. compared with 12 μg./ml.). On feeding the A-deficient rats either vitamin A or cocarboxylase, their blood pyruvate levels returned to normal; on the other hand, massive doses of aneurin (10 mg.) only reduced the levels to 24 μg./ml. It was thus concluded that vitamin A controls the phosphorylation of aneurin to cocarboxylase. Other brief reports of a vitamin A–B_1 synergism exist (Takagi & Saga, 1940; Mitalo, 1942; Cimino, 1944), but other investigators have found no interrelationship (Scheunert & Svreenivasa Rau, 1937; Scheunert & Wolfganger, 1938; Scheunert & Wagner, 1938; Dye, Porter & Kelly, 1947) and there is indeed one report that the two vitamins antagonize each other (Namikawa, 1940).

Because of the potential importance of the claims of von Euler & Högberg, and the contradictions in the literature, it was decided to repeat and extend their original investigation. In particular, assuming that the reported increases in blood pyruvate levels in vitamin A deficiency could be confirmed, it was important to find out whether the increase was due to pyruvate and not to α-ketoglutarate, the other main keto acid present in blood, for the method (Lu, 1939) used by von Euler & Högberg (1939, 1940), is not specific for pyruvate and would also measure the α-ketoglutarate present. A spectrophotometric method for determining both components simultaneously has been devised; it is based on the measurement at two wavelengths of the intensity of the colour produced by the 2:4-dinitrophenylhydrazones in alkaline solution. The preliminary extraction procedure follows closely, but not identically, that of Friedemann & Haugen (1943).

EXPERIMENTAL

Determination of blood pyruvate and α-ketoglutarate. The freshly drawn blood sample (about 1 ml.) is run into a 15 ml. centrifuge tube containing 2 ml. of 10% (w/v) trichloroacetic acid and centrifuged at 3000–3500 rev./min. for 5 min. The supernatant is decanted, a further 1 ml. of trichloroacetic acid added to the precipitate, the two mixed thoroughly and then recentrifuged. The supernatant is again collected, added to the first fraction and the combined extracts placed in a 25° bath; after temperature equilibration is complete (10 min.), 1 ml. of 0·1% (w/v, in 2N-HCl) 2:4-dinitrophenylhydrazine (also at 25°) is added. The mixture is incubated for 10 min., transferred quantitatively to a 25 ml. separating funnel and extracted with diethyl ether by shaking for 2 min. This procedure was found to give better reproducibility than the method recommended by Friedemann & Haugen (1943), who mixed the two phases by bubbling N_2 through them. This is not satisfactory when ether is the organic solvent, because excessive evaporation of the ether occurs, although it is quite satisfactory when toluene is used, as in the original method of Friedemann & Haugen (1943). In the present experiment, ether was preferred to toluene (Friedemann & Haugen, 1943) or ethyl acetate (von Euler & Högberg, 1939, 1940). The first solvent extracts only pyruvate, whilst the second, although extracting both pyruvate and α-keto-glutarate, is unsatisfactory because the degree of colour development varies from batch to batch (Long, 1942).

When the layers have completely separated, the aqueous layer is drawn off and discarded. The ethereal layer is then extracted with 6 ml. of 15% (w/v) Na_2CO_3 which extracts only the hydrazones of keto acids, leaving in the ether layer excess 2:4-dinitrophenylhydrazine and neutral hydrazones. The Na_2CO_3 layer is drawn off and 5 ml. transferred to a 10 ml. standard flask, which is then made up to volume with 1·5N-NaOH. A red colour quickly develops, which is then examined spectrophotometrically, using in the compensating cell a blank obtained by carrying out the full procedure on distilled water. In most experiments the blank was almost colourless, but occasionally a more marked colour appeared; this was generally traced to the sample of trichloroacetic acid in use at the time. The strength of the NaOH used in the development of the red colour is somewhat critical. The colour, especially that due to α-ketoglutarate, does not develop fully with N-NaOH, whilst it is unstable and rapidly fades in 3N-NaOH. With 1·5N-NaOH, development is complete and the colour is stable over 1–2 hr.

A colorimetric method for the simultaneous determination of the two keto acids in an extract prepared essentially as described above has been described by Friedemann & Haugen (1943); it is based on extinction measurements at two wavelengths, 420 and 540 mμ., and has given satisfaction in the hands of Coxon, Liébecq & Peters (1949), Buffa & Peters (1949) and Coxon & Peters (1950). The two main objections to this method are both related to the fact that the E values measured at 420 and 540 mμ. always differ considerably; this may lead (*a*) to error owing to the measurements being made on different regions of the extinction scale of a spectrophotometer; and (*b*) to lack of sensitivity owing to the E_{540} being so much less intense. One of the simplest ways of calculating the amount of each component in a binary mixture which possesses an isosbestic point is that described by Goodwin & Morton (1946). This method, which requires the measurement of the extinctions at two wavelengths, one being the isosbestic point, has been successfully applied to the present problem.

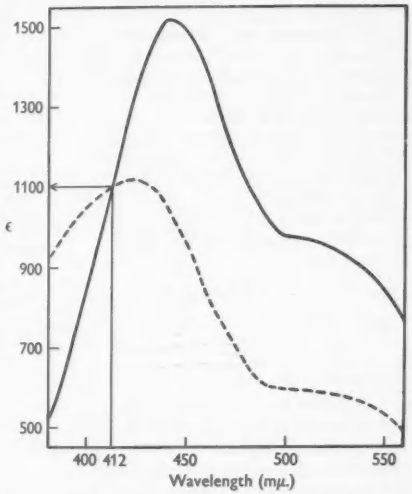

Fig. 1. The absorption spectra in 0·75 N-NaOH of the 2:4-dinitrophenylhydrazones of pyruvic acid and α-ketoglutaric acid. The isosbestic point (λ 412 mμ., ε 1100) is indicated ——, pyruvic acid; – – –, α-ketoglutaric acid.

The absorption curves of the 2:4-dinitrophenylhydrazones of pyruvic and α-ketoglutaric acids are recorded in Fig. 1. The pyruvic acid used was obtained by repeated fractional distillation under reduced pressure, the fraction distilling between 70 and 75° at 25 mm. Hg being used. The concentration of pyruvic acid in the solutions was determined by the bisulphite-binding power method of Clift & Cook (1932), it being impossible to weigh out accurately this intensely hygroscopic substance. The specimen of α-ketoglutaric acid used was kindly supplied by Prof. H. A. Krebs, F.R.S. It should be emphasized that the values recorded in Fig. 1 are obtained after putting the acids through the procedure just described; they were not obtained by dissolving the hydrazones directly in 0·75 N-NaOH. By referring all analyses to these standard curves, any slight systematic losses during the extraction procedure are eliminated.

The curves recorded here (Fig. 1) are somewhat different from those of Friedemann & Haugen (1943). This is because they did not use a spectrophotometer, but a colorimeter in conjunction with a series of filters; their curves, therefore, cannot be considered exact, for extinctions at wavelengths intermediate between those at which the filters transmitted maximally could not be measured.

It will be seen from Fig. 1 that the isosbestic point is at 412 mμ. and that the molar extinction (ε) at this wavelength is 1100. Taking into account the considerations discussed by Goodwin & Morton (1946), it was decided that maximal accuracy would be achieved by using as the second wavelength 440 mμ., where $\epsilon_{\text{pyruvic acid}}$ is 1515 (*A*) and $\epsilon_{\alpha\text{-ketoglutaric acid}}$ is 1055 (*B*). If x is the total molar concentration of keto acids and y the molar concentration of pyruvic acid, then, from the equations of Goodwin & Morton (1946), x is $E_{412\ \text{mμ.}}/1515$ and

$$y \text{ is } [E_{440\ \text{mμ.}} - 1055\ E_{412\ \text{mμ.}}/1100]/460.$$

Animals. In all experiments not involving the stock diet, weanling rats were used. In order to prevent coprophagy, by which means they could supplement their aneurin intake, animals on experiment were kept in separate cages of mesh recommended by U.S. Pharmacopoeia XII (see Hawk, Oser & Summerson, 1947).

Diets. Two experimental diets were used and their constituents are recorded in Table 1. Each animal received 10 g. diet/day. The animals receiving a sufficient amount of vitamin A + aneurin generally ate all their food; those on vitamin A and aneurin-deficient diets generally ate all their food until within 4–6 days of the end of the experiment, when, with the onset of deficiency symptoms, their appetite decreased. Diet no. 1 was made as near as possible to that of von Euler & Högberg (1939, 1940), although their published details are insufficient to be certain that the two diets were exactly the same in every detail. The uncertainties arose from the fact that von Euler & Högberg did not state (*a*) the iodine value (I.V.) of their hardened arachis oil, (*b*) the type of salt mixture used, or (*c*) the aneurin potency of the dried yeast. This last omission was particularly important, because it was likely to be a critical factor in the experiments.

Table 1. *Composition of the experimental diets*

(Diet no. 1 was made up as near as possible (as far as the information was available) to that used by von Euler & Högberg, 1940.)

Constituent	Diet no. 1 (parts by wt.)	Diet no. 2 (parts by wt.)
Casein (light white)	100	100
Gluten (wheat)	300	0
Hardened arachis oil (I.V. 32)*	65	0
Arachis oil (I.V. 120)*	0	30
Starch	0	325
Salt mixture* (Hawk & Oser, 1931)	25	25
Vitamin supplement*	See Table 2	

* von Euler & Högberg did not indicate the I.V. of their hardened arachis oil, the type of salt mixture used, or the potency of their vitamin supplement.

It was eventually concluded that their diet contained between 2·2 and 4·4 μg. aneurin/10 g., because they state that 100 μg. of aneurin plus 180 μg. of cocarboxylase represents 50–100 times the daily requirement of a growing rat. This was confirmed when, in a preliminary experiment, diet no. 1 was made up containing the amount of dried yeast used by von Euler & Högberg (1940), it was found to contain, by chemical analysis (kindly carried out by Dr E. Kodicek), 3·6 μg. aneurin/10 g. As the fat-soluble vitamins and those of the B complex are now available commercially in crystalline form, it was decided that a much better controlled experiment was possible using mixtures of these, rather than continuing to use dried yeast. The major components of the diet were, therefore, autoclaved at 15 lb. for 5 hr. to destroy any aneurin present in the diet (Elvehjem, Kline, Keenan & Hart, 1932). The appropriate amounts of vitamins (Table 2) were added daily to each 10 g. portion of diet. In addition to the vitamins recorded in Table 2, the following, often considered non-essential for rats (Brown & Sturtevant, 1949) were added: nicotinic acid (250 μg.), inositol (700 μg.), *p*-aminobenzoic acid (1 mg.) and 2-methyl-1:4-naphthoquinone (800 μg.); (the amounts are per 10 g. diet).

Table 2. *Vitamin allowance (other than aneurin) to rats on the experimental diets*

Vitamin	Allowance (μg./10 g. food)	Recommended allowance* (μg./day)
Pyridoxin	25	10
Calcium pantothenate	200	100
Choline chloride	10000	10000
Riboflavin	33	25
α-Tocopheryl acetate	800	300
Vitamin A	33	1·3

* Brown & Sturtevant (1949).

The stock diet consisted of rat cubes supplied by Lever Bros. and Unilever Ltd.

Collection of blood samples. In order to obtain sufficient blood for duplicate determinations, about 1 ml. is required; to obtain this amount it is necessary to kill the experimental animals. All the accepted routine procedures were unsuitable in this case, because any slight struggle of the animal raised its blood keto acid level considerably. Stotz & Bessey (1942), in their studies on blood pyruvate levels in rats, resorted to cord resection, whilst Handler (1945), using rabbits, found the use of light barbiturate anaesthesia most suitable. The latter procedure appeared more suitable for the present purpose, but it remained to be seen if the barbiturate which it was intended to use (veterinary nembutal supplied by Abbott and Co.) affected the blood pyruvate levels or the chemical determination or both. No effect was observed on the determination, but it was found that nembutal anaesthesia gradually reduces blood pyruvate levels (Goodwin & Williams, 1952). If, however, blood is drawn within 2–3 min. of injection of the anaesthetic, no significant error is involved.

The following procedure was found to be most effective: the rat, previously fasted for 18 hr., was injected intraperitoneally with 0·2 ml. of nembutal and then held firmly to prevent movement until anaesthesia was sufficiently advanced to allow the thorax to be opened. As soon as this was possible, the heart was exposed, the aorta cut, and the blood allowed to run into a 5 ml. beaker, which had been previously smeared with citrate. Citrate is the anticoagulant of choice because it tends to stabilize the pyruvate in the blood (Long, 1944), whilst oxalate causes rapid losses (Friedemann & Haugen, 1942). As quickly as possible, samples of the blood are poured into trichloroacetic acid, for in spite of the possible stabilizing action of citrate, there are many reports of the instability of pyruvate in blood (Wilkins, Weiss & Taylor, 1938; Bueding & Wortis, 1940; Bueding & Goodhart, 1941). The procedure is then as described under the section 'Determination of blood pyruvate and α-ketoglutarate' (p. 708).

RESULTS

The simultaneous determination of pyruvate and α-ketoglutarate levels in blood

Accuracy of the method. This was tested by preparing a series of known mixtures of the two components, treating them as if they were blood, and observing the analytical recovery. The results of a typical series are recorded in Table 3. It will be seen that, whatever the relative amounts of the two

Table 3. *Determination of pyruvate and α-ketoglutarate in prepared mixtures of the two*

Keto acids present in mixture (μg./ml.)		Molar ratio: pyruvate/ α-keto-glutarate	Keto acids found in mixture (μg./ml.)		Recovery	
Pyruvate	α-Keto-glutarate		Pyruvate	α-Keto-glutarate	Pyruvate (%)	α-Keto-glutarate (%)
7·9	32·0	0·415	8·2	31·8	104	99
16·0	33·3	0·81	16·0	32·2	100	97
7·9	16·0	0·83	7·8	16·1	99	101
4·9	7·5	1·08	5·1	7·5	104	100
24·4	25·0	1·59	25·4	22·2	104	89
7·9	8·0	1·66	8·3	7·1	105	89
29·4	20·0	2·42	29·7	20·6	101	103
8·2	4·2	3·24	8·4	3·3	102	79
7·9	4·0	3·32	7·8	4·6	99	115
7·9	4·0	3·32	7·8	3·9	99	98
9·8	2·0	8·3	10·0	1·1	102	55
29·4	5·0	9·7	30·4	5·8	103	116
44·0	5·0	14·5	44·0	8·7	100	174
9·8	0·0	∞	10·1	−1·0	103	—

components present, the accuracy of the pyruvate determinations is excellent, the mean recovery being 102 %. The accuracy of the α-ketoglutarate determinations is still good (mean recovery 101 %, with a slightly greater scatter than with pyruvate)

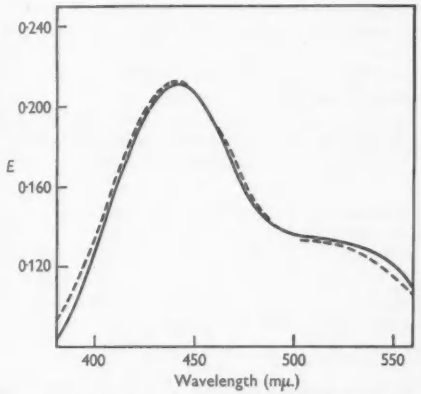

Fig. 2. The absorption spectrum in 0·75 N-NaOH of the 2:4-dinitrophenylhydrazones occurring in a specimen of normal rat blood, compared with that calculated according to the procedure outlined in the text, assuming only pyruvic and α-ketoglutaric acids are present. ——, observed curve; – – –, calculated curve.

provided that the molar ratio pyruvate:α-ketoglutarate is below 4. When this ratio is above 4, then the determination of α-ketoglutarate ceases to be reliable. As this ratio has never yet been observed in blood, this failure is of no practical consequence. If the blood filtrates contain keto acids other than pyruvate and α-ketoglutarate, then this method is not applicable. There appears to be no reasonable doubt, however, that in normal blood these two ketoacids are the only ones present in detectable amounts (Friedemann & Haugen, 1943). The recent report (Calvallini, Frontali & Toschi, 1949) that four 2:4-dinitrophenylhydrazones arise from blood, still requires confirmation, for there is evidence that two of the 'spots' observed on the Italian workers' chromatogram are artifacts (see, for example, Altmann, Crook & Datta, 1951). As a final check of the assumption that only two keto acids are involved, the following experiment was carried out: The complete absorption spectrum of the colour produced by the hydrazones extracted from a sample of blood was plotted. Assuming that only the two reported constituents were present, the amounts of these were calculated in the usual way. The complete spectrum which would be expected from these results was then computed and compared with the observed curve. It will be seen (Fig. 2), that the two curves are, within experimental limits, coincident over the spectral range examined. If any other keto acids had been contributing to any significant extent (e.g. > 1 %) to the observed spectrum, marked divergencies between this and the calculated curve would have been encountered.

Blood pyruvate and α-ketoglutarate levels on various diets

Stock diet. The mean value of the pyruvate and α-ketoglutarate levels of rats of both sexes, drawn at random at different times of the year from the stock colony, are recorded in Table 4. It will be seen that there is no significant difference between the sexes, although von Euler, von Euler & Pettersson (1941), claimed that increased pyruvate levels were observed at oestrus.

Experimental diets. The results obtained in a typical experiment using a diet (diet 1, Table 1) simulating as nearly as possible that of von Euler & Högberg (1939, 1940) are recorded in Table 5. Weanling rats were used and the experiment continued until the group on the vitamin A-deficient diet began to lose weight (34–38 days). That this was a true sign of the onset of vitamin A deficiency was confirmed by analysing the livers of these rats for vitamin A; in no case, using the antimony trichloride method, could vitamin A be detected. It will be seen from Table 5 that no significant differences in the blood levels of either keto acid occurred between the two groups. A statistical comparison between these results and those obtained with animals on the stock diet indicates that, in both groups, the two parameters are significantly higher than those of the normal diet ($P < 0{\cdot}001$ in both cases).

Table 4. *Mean values for pyruvate and α-ketoglutarate of blood in male and female rats chosen at random and maintained on a stock diet*

Sex	No. of animals	Pyruvate μg./ml.	Pyruvate S.D.	Pyruvate P	α-Ketoglutarate μg./ml.	α-Ketoglutarate S.D.	α-Ketoglutarate P
Mixed	20	9·14	∓2·31	—	5·76	∓2·70	—
Male	8	8·36	∓1·41	0·10–0·20	6·49	∓2·15	0·10–0·20
Female	8	9·94	∓2·63		4·70	∓2·01	

Table 5. *Mean blood levels of total keto acids, pyruvate and α-ketoglutarate in weanling rats on experimental diets*

(The rats were maintained for 34–36 days on diet 1 (Table 1), supplemented with 3·5 μg. aneurin/day, Group *A* was given vitamin A, group *B* was not.)

	Group *A* (5 rats)		Group *B* (4 rats)		*P* (between groups)
	(μg./ml.)	S.D.	(μg./ml.)	S.D.	
Total keto acids	26·7*	∓5·2	29·7*	∓9·3	0·50–0·70
Pyruvate	13·9	∓3·2	16·2	∓5·1	0·30–0·50
α-Ketoglutarate	15·8	∓4·4	16·2	∓5·3	0·80–0·90

* Total keto acids are expressed as μmoles/100 ml.

Table 6. *Mean blood levels of total keto acids, pyruvate and α-ketoglutarate in groups of weanling rats (six per group) fed diet 2 (Table 1) and varying supplements of aneurin*

Aneurin intake (μg./day)	Total keto acids (μmoles/100 ml.)	S.D.	*P*	Pyruvate (μg./ml.)	S.D.	*P*	α-Ketoglutarate (μg./ml.)	S.D.	*P*
4	22·5	∓7·1	0·50–0·70	12·7	∓5·1	1·0	12·4	∓7·1	0·30–0·50
6	20·6	∓7·3	0·50–0·70	12·7	∓6·7	0·80–0·90	8·9	∓3·8	0·70–0·80
8	18·6	∓6·8	0·05–0·10	12·1	∓2·9	0·001–0·01	9·7	∓5·2	0·001–0·01
10	13·0	∓1·9	0·10–0·20	7·9	∓0·6	0·90	6·0	∓2·7	0·10–0·20
12	14·9	∓1·1		8·0	∓1·5		8·5	∓1·5	

When it was found impossible to reproduce the effect attributed by von Euler & Högberg (1939, 1940) to vitamin A deficiency, it was decided to carry out further similar experiments on a more balanced diet, for the diet used by these workers is virtually carbohydrate free. Using diet 2 (Table 1), a preliminary investigation was carried out to determine the aneurin intake at which blood keto acid levels begin to rise. It was thought that if the observations of von Euler & Högberg (1940) were due to an increased aneurin requirement in vitamin A deficiency, then the best way to demonstrate this would be to observe the blood levels of a group of rats fed a vitamin A-deficient diet containing just sufficient aneurin to prevent elevated blood keto acid levels on the same diet supplemented with vitamin A.

The results obtained on diets containing varying amounts of aneurin are recorded in Table 6. The growth curves for the various groups are given in Fig. 3. It will be seen from Table 6 that there is a significant difference between the blood levels of keto acid of rats receiving 8 μg. aneurin/10 g. diet and those receiving 10 μg. This conclusion is strengthened when it is noted that there is no statistical difference between the values obtained for rats on the stock diet and those on the diets containing 10 and 12 μg. aneurin/10 g., whilst the values for the 4, 6 and 8 μg. aneurin/10 g. diets are all significantly higher than those on the stock diet. Similarly with α-ketoglutarate levels, there is a significant difference between the 8 and 10 μg. diet. An apparent inconsistency arises here, for the α-ketoglutarate levels on the 12 μg. diet are above those on the 10 μg. diet and not significantly different from those on the 8 μg. diet. As one extremely high value was encountered in this group, no real significance can be attached to this apparent anomaly, and in fact, the 10 and 12 μg. values, when compared with values on the stock diet, are found not to be significantly different from normal. The growth curves (Fig. 3) confirm the findings on blood; it will be seen that after 30 days on the various diets, only those animals obtaining 10 and 12 μg.

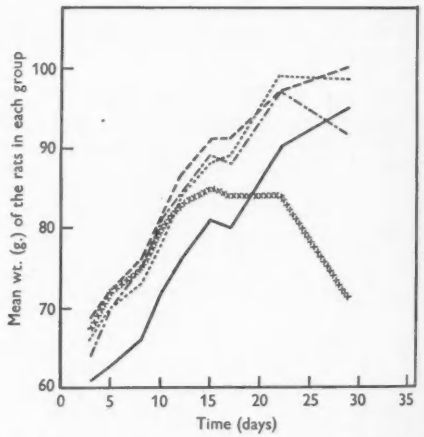

Fig. 3. The mean growth rates of groups of weanling rats maintained on diet 2 (Table 1) supplemented with varying amounts of aneurin. × × ×, 4 μg. aneurin/day; –·–·–, 6 μg. aneurin/day; ·····, 8 μg. aneurin/day; – – –, 10 μg. aneurin/day; ——, 12 μg. aneurin/day.

aneurin/10 g. diet, continue to gain weight; those on the lower levels begin to lose weight with varying rapidity after between 20 and 25 days on the diet.

As a result of this experiment it was decided to examine the effect of vitamin A deficiency on the blood keto acid levels of rats maintained on diet 2 (Table 1) and supplemented with either 8 or 10 μg. aneurin/10 g.diet. Two groups of weanling rats were taken; one was given vitamin A and the other deprived of vitamin A. Each of these groups was divided into two sub-groups, one of which obtained 8 μg., and the other 10 μg. aneurin/10 g. diet. The experiment was continued until vitamin A deficiency was established as evidenced by cessation of growth followed by a decline in weight. The absence of vitamin A from the livers of the deficient groups was demonstrated in the usual way using the antimony trichloride method.

acid levels occur on this diet although it is virtually carbohydrate-free, and Banerji (1941) and Dann (1945) found that clinical signs of avitaminosis-B_1 did not occur on carbohydrate-free diets even in the complete absence of aneurin from the diet. They did, however, observe that in the absence of aneurin, rats on carbohydrate-free diets excreted more bisulphite-binding substances (keto acids) in the urine. Unfortunately, neither investigator measured the blood keto acid levels, but one would suppose that they would also be raised if increased urinary excretion of bisulphite-binding substances occurs. The present experiments on carbohydrate-free diets indicates that this is so, and that on a carbohydrate-free diet, a daily intake of 3·5 μg. of aneurin is insufficient to prevent elevated blood keto acid levels although no typical clinical signs of aneurin deficiency existed. It is also important to notice

Table 7. *Mean blood levels of total keto acids, pyruvate and α-ketoglutarate fed diet no. 2 with either 8 or 10 μg. aneurin/day, and with or without vitamin A*

(Groups of weanling rats, five per group were used.)

	Aneurin intake 8 μg./day					Aneurin intake 10 μg./day				
	No vitamin A supplied		Vitamin A supplied			No vitamin A supplied		Vitamin A supplied		
	(μg./ml.)	S.D.	(μg./ml.)	S.D.	*P* (between groups)	(μg./ml.)	S.D.	(μg./ml.)	S.D.	*P* (between groups)
Total keto acids	16·0*	∓2·7	15·0*	∓5·6	0·70–0·80	13·1*	∓3·2	13·2*	∓1·8	>0·90
Pyruvate	11·2	∓2·6	10·9	∓4·8	>0·90	10·4	∓2·1	10·0	∓2·6	0·80–0·90
α-Ketoglutarate	4·8	∓2·9	3·9	∓0·75	0·50–0·70	2·1	∓1·6	2·6	∓1·5	0·50–0·70

* Total keto acids are expressed as μmoles/100 ml.

It will be seen from the results recorded in Table 7 that no significant differences are apparent between the groups deficient in vitamin A and those receiving supplements of this vitamin. It is interesting also to note in this experiment that there were no significant differences between the blood keto acid levels in the groups fed 8 and 10 μg. aneurin, the values on the 10 μg. level being increased above normal. This indicates how close to the minimum daily requirement of aneurin this value is; the rise on the 10 μg. diet may be due to the fact that the duration of this experiment was longer, 40 days compared with 30 days in the previous experiment.

DISCUSSION

Using a diet as comparable as possible with that of von Euler & Högberg (1939, 1940), it has not been possible to demonstrate that vitamin A deficiency has any effect on the blood levels of either pyruvate or α-ketoglutarate. On this diet, the keto acid levels were significantly raised compared with normals, but the increases were the same, irrespective of whether the rats were deficient in vitamin A or not. It is interesting to note that increases in blood keto

that the rise of α-ketoglutarate levels is relatively greater than that of pyruvate; this may well be due to the high content of gluten which contains considerable amounts of glutamic acid which could, by deamination, give rise to α-ketoglutaric acid. Oberdisse (1947) has shown that in dogs intravenous injection of alanine causes an increase of blood pyruvate. Using a normal carbohydrate-containing diet supplemented with borderline amounts of aneurin, it was also not possible to demonstrate any effect of vitamin A deficiency. These latter experiments must be considered critical, for any effect of vitamin A on aneurin metabolism should have revealed itself in these experiments. It must be concluded that the higher pyruvate levels recorded by von Euler & Högberg (1939, 1940) were not due to vitamin A deficiency; consequently their evidence that vitamin A controls the phosphorylation of aneurin to cocarboxylase cannot be valid.

It is obvious from the results recorded in Tables 6 and 7 and in Fig. 3 that the minimal aneurin requirement for growing rats lies between 8 and 10 μg./day; this confirms previous estimates, e.g. Arnold & Elvehjem (1938), 10 μg.; Yudkin (1941), 8 μg. It is also not inconsistent with the only previous report

(Kratzing & Slater, 1950) on the maximal aneurin intake on which hyperpyruvaemia was observed, the figure given being 7·8 μg./day.

SUMMARY

1. A spectrophotometric method for the simultaneous determination of blood pyruvate and α-ketoglutarate levels is described.

2. Using a carbohydrate-free diet essentially similar to that of von Euler & Högberg (1940), the claims of these authors that vitamin A deficiency increases blood pyruvate levels in rats was not confirmed; α-ketoglutarate levels were also unaffected.

3. Using a diet containing a normal proportion of carbohydrate and supplemented so as to meet the minimal aneurin requirements of growing rats, it was again impossible to demonstrate any effect of vitamin A deficiency on blood pyruvate and α-ketoglutarate levels.

4. On a carbohydrate-free diet a daily intake of 3·5 μg. of aneurin is insufficient to prevent raised blood pyruvate and α-ketoglutarate levels, irrespective of the vitamin A status of the animals.

5. On a normal diet, the minimal daily aneurin intake required to prevent increased blood pyruvate and α-ketoglutarate levels in rats is between 8 and 10 μg.

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The Immunological and Serological Properties of Phosvitin

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In view of the pronounced influence of polar groups in the molecule in determining the specificity of protein antigens, it might be expected that the phosphate groups in natural and artificial phosphoproteins would be largely responsible for the immunological specificity of these proteins. Investigation of their serological properties might therefore yield useful information about the structure, and the biosynthesis, of natural phosphoproteins.

Heidelberger, Davis & Treffers (1941) have investigated the immunological properties of artificially phosphorylated ovalbumin, and Mayer & Heidelberger (1946), and Boursnell, Dewey, Francis & Wormall (1947) those of artificially phosphorylated serum proteins. Kesztyus & Kocsis (1942) have also examined the immunological properties of casein after dephosphorylation with dilute alkali, and compared these properties with those of the same material after rephosphorylation with phosphorus oxychloride ($POCl_3$), and with those of casein.

In all these cases, however, interpretation of the results is complicated by the unknown factor of the degree of change effected in the protein molecule by the somewhat drastic chemical treatment to which it has been subjected.

The investigations of Francis & Wormall (1948, 1950) on the immunological properties of lipovitellin were directed mainly towards elucidating the respective parts played by the phospholipin and the phosphoprotein of the antigen complex, but the discovery by Mecham & Olcott (1949) of the new phosphoprotein, 'phosvitin,' in hen's egg yolk, has rendered it desirable that these investigations should be extended in order to obtain more information on the nature of the protein part of the complex.

Phosvitin is a protein of relatively low molecular weight, containing about 10% P, and accounting for at least 60% of the protein P of egg yolk. Mecham & Olcott (1949) infer that most, if not all, of the P of vitellin is due to phosvitin.

Experiments have therefore been undertaken on the exchange of radioactivity, *in vitro*, between lipovitellin and a ^{32}P-containing phosvitin preparation, and the immunological and serological properties of phosvitin itself have also been investigated.

MATERIALS AND METHODS

Lipovitellin solutions. These were prepared as described by Francis & Wormall (1948).

Phosvitin solutions. These were prepared as described by Mecham & Olcott (1949). Some of these preparations were made from the yolks of eggs laid by a hen 3–6 days after it had received an intramuscular injection of 2 mc. ^{32}P as Na_2HPO_4.

Phosphorylated proteins. Phosphorylated proteins were obtained, as described by Boursnell, Dewey & Wormall (1948), by the action of $POCl_3$ (0·5 g. $POCl_3$/g. protein) on human serum or plasma, rabbit and chicken serum, and on the mixed globulins obtained from horse serum.

Alum-precipitated antigens. Some of the antigens were prepared following the method of Proom (1943). Approximately 2% solutions of phosphorylated human serum proteins and of phosvitin were treated with equal volumes of 10% (w/v) potassium alum. The pH was adjusted to 6·5 with 5N-NaOH, and the precipitate centrifuged, washed with 0·9% (w/v) NaCl containing 0·02% merthiolate and finally made up with merthiolated saline to the same volume as the original phosphoprotein solution.

Antisera. Antisera to ethanol-ether extracted, lipid-free vitellin were prepared as described by Francis & Wormall (1948). These antisera react well with lipovitellin, but contain somewhat less of the antibodies reacting principally with the phospholipin part of the lipoprotein complex, than do antisera to lipovitellin. Only aged sera, which had lost these 'antiphospholipin' antibodies (Francis & Wormall, 1950), were used in the experiments reported here. Antisera to phosphorylated human serum proteins were obtained by intramuscular injection of 5 ml. of the alum-precipitated antigen into each hind leg of a rabbit, at intervals of 10–12 days, satisfactory antisera usually being obtained 10 days after the second injection. Sera were also collected from rabbits which had received courses of injection of phosvitin, (*a*) by intramuscular injection of 10 ml. of alum-precipitated antigen suspension at intervals of 10 days, (*b*) by intravenous injection of 2 ml. of approx. 2% phosvitin solution, or (*c*) by intraperitoneal injections of 5 ml. of 2% phosvitin solution, at weekly intervals for 1 month. No differences were observable in the results with the sera obtained by the three methods.

Quantitative precipitin reactions. The antiserum (usually 0·5–1 ml.) was mixed with antigen solution (0·5–3 ml.) and sufficient 0·9% NaCl solution to bring to a standard volume. When phosvitin was used as an inhibitor it was added to the antiserum before the antigen. When lipovitellin was used as antigen, solid NaCl was added (0·1 g./ml.) to the antiserum and to the phosvitin solution, and 10% (w/v) NaCl was

substituted for 0·9% NaCl, to prevent precipitation of lipovitellin and to minimize non-specific precipitation by phosvitin. The mixtures were kept at 37° for 30 min.–1 hr., overnight at 0–4°, and centrifuged at 0°. The precipitates were then washed three times with ice-cold 0·9 or 10% NaCl, dissolved in 0·5 ml. 0·1 N-NaOH, and transferred to micro-Kjeldahl flasks for incineration.

Complement fixation tests and qualitative precipitin tests. These were carried out as described by Berenblum & Wormall (1939) and Hopkins & Wormall (1933) respectively. When lipovitellin was used as antigen for complement fixation tests, the progressive threefold dilutions with 0·9% NaCl were made from a fresh 0·05% suspension in 0·5% NaCl, obtained by diluting with water a 0·25% solution in 2·5% NaCl.

Total N *determinations.* These were carried out as described by Banks, Francis, Mulligan & Wormall (1951).

Total P *determinations.* Colorimetric P determinations were made by King's (1932) modification of the method of Fiske & Subbarow (1925).

Non-lipid or 'protein' P determinations. The lipovitellin solution (containing about 0·1 mg. P) was pipetted into 10 vol. 7:3 ethanol-ether mixture. The precipitate was centrifuged, washed twice with 5 ml. ethanol-ether mixture, dried in a current of warm air and incinerated with 1·2 ml. 60% $HClO_4$.

Radioactivity determinations. These were made by evaporating the radioactive solutions, together with 3 drops of a 10% suspension of kaolin in 50% ethanol, on 14 mm. diameter nickel planchettes. The dried preparations were then counted under a standard 'bell'-type counter with a Panax amplifier and scaler unit.

solution in presence of ^{32}P-containing phosvitin should contain ^{32}P. If the percentage of phosvitin originally present in the lipovitellin is unchanged by this treatment, no change in the ratio of protein P to total P should result; whereas any radioactivity in the precipitate due to co-precipitation of unchanged, non-radioactive, lipovitellin and ^{32}P-containing phosvitin would be accompanied by a corresponding increase in this ratio.

Two 12 ml. portions of an approximately 1% solution of lipovitellin in 10% NaCl were each mixed with 1·2 ml. of an approximately 1% solution of ^{32}P-containing phosvitin, and immediately poured into 240 ml. water. The precipitated lipovitellin was separated by centrifugation, redissolved in 6 ml. of 20% NaCl solution and again poured into water (240 ml.). The solution and reprecipitation were repeated twice more. After the second and third precipitations, portions of the precipitates (approx. one-third and one-half respectively) were reserved for analysis, and the volumes of 20% (w/v) NaCl and water were reduced in proportion at each subsequent reprecipitation. The twice, thrice and four times precipitated samples of lipovitellin were then dissolved separately in 1 ml. 0·1 N-NaOH, transferred quantitatively to measuring flasks, and made up to 3 ml. Duplicate determinations of radioactivity, total P and non-lipid P were then made on 0·4, 0·4 and 0·6 ml. samples of each solution. A third sample of lipovitellin was treated in the same way, but without the addition of phosvitin, and analysed after four precipitations. This repeated reprecipitation caused no measurable change in the ratio of protein P to total P.

Table 1. *Exchange of radioactivity between lipovitellin and* ^{32}P*-containing phosvitin*

(System: Lipovitellin in 10% NaCl solution, 12 ml. (1·588 mg., total P, 0·647 mg. (40·7% of total) protein P). ^{32}P-containing phosvitin, 1·2 ml. (1·24 mg. P = 65·6% of total protein P in mixture). Lipovitellin precipitated from mixture by water (240 ml.), redissolved in 20% NaCl and reprecipitated, four times in all. The letters (*a*) and (*b*) refer to separate experiments.)

	No. of precipitations	Protein P (as % of total P)		Lipovitellin protein P replaced by radio-phosvitin P* (%)
		Non-lipid P (total protein P)	Radio phosvitin P	
Lipovitellin	1–4	40·7	0·0	—
Lipovitellin + phosvitin	2	(*a*) 51·4	37·1	57·1
		(*b*) 50·0	30·1	42·0
	3	(*a*) 46·8	26·7	45·0
		(*b*) 43·8	25·1	51·6
	4	(*a*) 44·3	24·9	49·2
		(*b*) 45·4	25·8	47·8

* These figures were calculated after making allowance for the additional phosvitin carried down by the precipitate; had there been complete exchange, all figures in this column would be 65·6.

RESULTS

The exchange of radioactivity between lipovitellin and ^{32}P*-containing phosvitin*

If any part of the phosphorus in a lipovitellin preparation is due to the presence of phosvitin, which dissociates from it in solution in 10% NaCl and recombines when the lipovitellin is reprecipitated, then lipovitellin precipitated from 10% NaCl

The results of the two experiments (Table 1) show that there is a very rapid interchange under the conditions of the experiment. The percentage of protein P in the precipitate is somewhat greater than that in the original lipovitellin, particularly after only two precipitations. This is apparently due to some additional phosyitin adhering strongly to the precipitate; but even after allowance has been made for this additional phosvitin P, 48% of the

remaining protein P in the precipitate was derived from the originally free phosvitin, compared with a figure of 65·6 % theoretically possible if there had been complete equilibration between the phosvitin P and vitellin P. In order further to eliminate the possibility of the result being due to adsorption, or combination of phosvitin with the precipitated lipovitellin, the experiment was repeated using equal volumes of 1 % solutions of lipovitellin and radioactive phosvitin, and again the radioactivity of the separated lipovitellin was about 75 % of the maximum theoretically possible.

The action of phosphoprotein antigens on 'antiphosvitin' sera

Qualitative precipitin tests on 'antiphosvitin' sera, using phosvitin as antigen, showed no visible precipitate after 1 hr. at 37°. When either lipovitellin or phosphorylated horse serum globulin were used as antigen, however, a very faint precipitate was discernible in most cases. In quantitative precipitin reactions, 2 ml. portions of several different sera were treated with lipovitellin or phosphorylated globulins to give maximal precipitation as nearly as could be judged. In no case was more than 9 μg. nitrogen precipitated by phosphorylated protein, or 5 μg. nitrogen by lipovitellin, per ml. serum. (In one case, 1 ml. of 'antiphosvitin' serum treated with 0·1 ml. 0·25 % phosvitin gave a precipitate containing 25 μg. nitrogen, but in view of later experiments it is considered that this was probably due to non-specific precipitation.) Control experiments were carried out with normal rabbit serum in place of 'antiphosvitin', and although no visible precipitates were obtained, the tubes were treated exactly as before. The total nitrogen in these 'precipitates' was never greater than 2·5 μg./ml. serum.

No evidence of complement fixation could be obtained when lipovitellin or phosvitin were mixed with 'antiphosvitin' sera in presence of guinea pig serum, although the lipovitellin gave strong precipitin and complement fixation reactions with antivitellin serum.

The action of phosvitin on antisera to phosphoproteins

Qualitative precipitin tests were carried out with phosvitin as test antigen, using antisera to phosphorylated proteins and to vitellin. Complement fixation tests were also carried out with the phosvitin-antivitellin system, but in no case was any reaction discernible. (All the phosvitin preparations tested were found to be slightly 'anti-complementary', possibly due to combination or co-precipitation occurring between phosvitin and a protein component of complement.)

The effect of phosvitin on phosphoprotein precipitin systems

Although phosvitin does not precipitate antibodies to phosphorylated proteins or to vitellin, this does not preclude the possibility that it might react with them. Experiments were therefore carried out to determine whether phosvitin had any inhibitory effect on precipitin reactions between these antibodies and appropriate antigens.

Table 2. *Effect of phosvitin on the phosphorylated protein precipitin system*

(System: 1 ml. rabbit antiserum to phosphorylated human serum proteins; 0·5 ml. phosphorylated horse globulin solution (200 μg. N), 0·9 % NaCl added to each tube to give a total volume of 3·5 ml. after addition of phosvitin.)

	Total N in precipitate (μg.)			
Phosvitin added (μg. N)	Complete system	Control with no antiserum	Control with no antigen	Control with normal serum
0	62	—	—	—
144	58	4	5	5
288	62	5	6	7
576	97	4	17	20

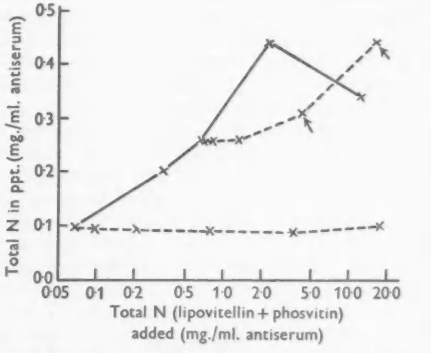

Fig. 1. The effect of addition of phosvitin to lipovitellin-antivitellin-precipitin systems. The dotted lines denote the addition of phosvitin, continuous lines the addition of lipovitellin, to 1 ml. of antivitellin serum. In control experiments corresponding to the points marked with an arrow, using normal rabbit serum instead of antivitellin serum, large and variable amounts (0·18–0·4 mg. N) were obtained, due to non-specific precipitation by phosvitin.

(*a*) *Phosphorylated proteins systems.* The effect of phosvitin on the precipitation of an antiserum to phosphorylated human serum proteins by phosphorylated horse serum globulin, phosphorylated rabbit serum proteins and phosphorylated human

plasma proteins, was investigated qualitatively. In these experiments quantities of phosvitin up to four times the weight of antigen used were added to the precipitin systems, but in no case could inhibition be detected by visual inspection. In some cases, however, there appeared to be an increase in the amount of precipitate when the larger amounts of phosvitin were used. A quantitative experiment was therefore carried out, the results of which are shown in Table 2. These figures show no evidence of inhibition by phosvitin. The increased precipitation when the largest amount of phosvitin was used is found also in the control systems, and is therefore almost certainly due to non-specific co-precipitation between phosvitin and normal serum proteins.

(*b*) *The lipovitellin-antivitellin system.* Quantitative precipitin reactions were carried out in which (i) varying amounts of lipovitellin, and (ii) varying amounts of phosvitin together with a fixed amount of lipovitellin, were added to a fixed volume of an aged antiserum to vitellin. The results are shown in Fig. 1. Again there is no evidence of inhibition, but an increase in the amount of precipitate due to non-specific precipitation was obtained when large amounts of phosvitin were added.

DISCUSSION

The artificial phosphorylation of proteins by treatment with phosphorus oxychloride (Bechold, 1901; Neuberg & Pollak, 1910; Neuberg & Oertel, 1914; Rimington, 1927) is always accompanied by some denaturation, and probably causes significant changes in the protein molecule besides the introduction of phosphate groups. The changes in the immunological properties of ovalbumin caused by this treatment (Heidelberger *et al.* 1941) may therefore be due to a variety of causes. Phosphorylation of serum proteins (Mayer & Heidelberger, 1946; Boursnell *et al.* 1947, 1948) results in products whose phosphate groups are fairly rapidly lost under physiological conditions of temperature and pH, but these products, when injected into rabbits, in some of our experiments, produced antisera still capable of reacting with other artificially phosphorylated proteins. In the present series of investigations, in an attempt to retain the intact phosphorylated serum proteins in the tissues for a longer time after injection, the technique of intramuscular injection of alum-precipitated antigens (Proom, 1943) has been used. The injection of alum-precipitated phosphorylated human serum proteins has resulted in the production of antisera reacting weakly with phosphorylated rabbit serum, chicken serum and horse serum globulins, but these antisera do not react, either in precipitin or complement fixation reactions, with phosvitin or lipovitellin. Furthermore, the addition of phosvitin to a phosphorylated protein precipitin system does not result in any diminution in the amount of precipitate obtained, although it might be expected that phosvitin, even though it does not precipitate antibodies to phosphoprotein, would be capable of inhibiting formation of a specific precipitate in any system where the primary reaction was due to the presence of phosphate groups in the antigen.

These results therefore indicate that very little, if any, of the immunological properties of artificially phosphorylated proteins are likely to be due directly to the introduction of phosphate groups into the molecule.

Francis & Wormall (1948, 1950), have shown that the lipoprotein complex, lipovitellin, on injection into rabbits, produces antisera capable of specific precipitation with lipovitellin; the specificity of the antibodies produced is, however, directed more towards the phospholipin part of the complex than the phosphoprotein part. These antisera, on ageing, lost much of their antibodies reacting primarily with lipids, and thereafter precipitated phospholipin and phosphoprotein in approximately the same ratio as that in the original antigen. Owing to the insolubility of vitellin, however, it was impossible to use the lipid-free antigen in quantitative precipitating reactions, and therefore it could not be ascertained what parts the phosphoprotein and phospholipin played respectively in the precipitin reaction with these aged antisera.

If the inference of Mecham & Olcott (1949) regarding the identity of the phosphorus of vitellin and phosvitin is correct, this difficulty should be overcome by using phosvitin in place of vitellin. However, the use of phosvitin as a test antigen is complicated by the fact, observed by these authors, that it possesses the property of precipitating non-specifically with other proteins, e.g. protamine and bovine serum albumin. These precipitates are soluble in 2N-sodium chloride, like lipovitellin, but precipitate at lower salt concentrations.

The increase in the amount of precipitate in a lipovitellin-antivitellin system, on adding relatively large amounts of phosvitin, is almost certainly due to this non-specific precipitation, and the failure to cause either increase or inhibition at lower phosvitin concentrations indicates that phosvitin plays little or no part in the immunological reactions of lipovitellin.

This is further confirmed by the finding that phosvitin and antivitellin serum incubated with guinea pig serum showed no fixation of complement, although the same antiserum reacted strongly, in both precipitin and complement fixation tests with lipovitellin.

The conditions used by Mecham & Olcott (1949) in their isolation of phosvitin, involving nothing

more drastic than treatment with 0·4 M-magnesium sulphate and saturated ammonium sulphate, are not such as would lead one to expect degradation of the natural phosphoproteins of the egg yolk. Their inference that the protein P of samples of vitellin is largely, if not entirely, due to the presence of small amounts of phosvitin is, on these grounds, not unreasonable. On the other hand, Osborne & Campbell (1900) have reported the isolation from peptic digests of vitellin of a product, in 25 % yield, containing all the vitellin P, and in addition, 1 % of sulphur. Since phosvitin is resistant to peptic digestion and contains no sulphur, it could not have been the mother substance of this product, and it appears unlikely that this product, which contained 3–4 % P, could have been phosvitin contaminated with twice its weight of P-free protein. Other workers (Levene & Alsberg, 1900; Swigel & Posternak, 1927*a*) have reported the isolation of vitellin degradation products of high P-content similar to phosvitin, and Swigel & Posternak (1927*b*) have also reported the presence in egg yolk of an enzyme capable of liberating polypeptides of high P-content from vitellin. On these grounds, therefore, the possibility of phosvitin being a degradation product of vitellin cannot be entirely excluded. Neither vitellin nor phosvitin are believed to be homogeneous proteins, and the composition of different lipovitellin preparations certainly varies slightly. However, a lipovitellin preparation which has been several times reprecipitated is unlikely to contain an enzyme which could produce phosvitin from it sufficiently rapidly to account for the exchange reaction reported here, and at the same time leave most of the original material still in a readily precipitable form. It therefore seems highly improbable that such a preparation would undergo exchange of P with a phosvitin preparation unless the lipovitellin did actually contain phosvitin. In the experiments reported here, this exchange did occur, to the extent of about 75 % of that theoretically possible if all the vitellin P were due to phosvitin, and the same result was obtained whether the ratio of phosvitin P to vitellin P in the mixture was 2:1 or 20:1, and whether the lipovitellin was precipitated immediately after mixing, or after incubating at 37° for 1 hr.

The lipovitellin precipitated from a lipovitellin-phosvitin mixture contained more protein P, as a percentage of the total P (45 % as against 41 %) than did the original lipovitellin, indicating that some phosvitin had combined fairly firmly with the vitellin part of the lipoprotein complex. This additional protein P is, however, incapable of accounting for more than a fraction of the radioactivity in the precipitate, most of which must therefore have arisen from an exchange of P between the two components of the system. It is inconceivable that the phosphate groups of the two proteins should have exchanged under the experimental conditions employed, and the result therefore confirms Mecham & Olcott's inference, i.e. about 75 % of the protein P of vitellin appears to be due to phosvitin.

No antisera have yet been found to give specific precipitates with phosvitin, neither does phosvitin act as a specific inhibitor with lipovitellin or phosphorylated protein precipitin systems. It is therefore hardly surprising that it is incapable of producing antisera when injected into rabbits. This lack of antigenicity might be due to its relatively simple structure, and like gelatin, the molecule may lack the rigidity which appears to be necessary for antigenicity. On the other hand, it is quite possible that, since a large proportion of the molecule (about 50 %) is composed of phosphoserine groups, the structure of the remainder of the molecule does not differ sufficiently from that of phosphoproteins of similar function which presumably also occur in other species of animal. In this connexion it should be noted that Harris (1946) has reported the occurrence of a phosphoprotein in frog spawn, and Roepke & Bushnell (1936) have detected by immunological methods traces of a protein, similar to vitellin, in the serum of cocks, and more in that of laying hens. In the experiments reported here, twelve rabbits were used for the preparation of 'antisera' to phosvitin, six of either sex, and no differences were observed between the behaviour of the males and females.

The apparent traces of a precipitin reaction obtained in a few cases between 'antiphosvitin' sera and lipovitellin or phosphorylated proteins, which could not be confirmed in complement fixation tests, were probably not significant, and might have been due to traces of some impurity, such as a denatured protein, giving reactions with some non-specific antibody present in the serum.

The antigenicity of vitellin would therefore appear to be due to the non-phosvitin component of the protein.

SUMMARY

1. Phosvitin containing ^{32}P has been prepared, and shown to undergo an exchange reaction with lipovitellin in 10 % sodium chloride solution, to the extent of about 75 % of that theoretically possible if all the phosphorus of the vitellin were due to phosvitin.

2. Phosvitin has been shown not to react, either in precipitin or complement fixation reactions, with antisera to artifically phosphorylated proteins or to vitellin.

3. Phosvitin does not inhibit precipitate formation in lipovitellin-antivitellin or phosphorylated protein precipitin systems.

4. Injection of phosvitin, either intravenously or intraperitoneally, or intramuscularly in the form of an alum precipitate, into male or female rabbits does not produce sera capable of reacting in precipitin or complement fixation reactions with phosvitin, lipovitellin, or artificially phosphorylated proteins.

5. The significance of these results is discussed, in relation to the composition of vitellin and the structure of phosvitin.

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